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MAMMALIAN SOMATIC CELL FUSION IN VIVO

by

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled "Mammalian Somatic Cell Fusion In Vivo" submitted by Herbert William Janzen in partial fulfillment of the requirements for the degree of Master of Science (Surgery).

ABSTRACT

Somatic cell fusion *in vitro* is a well established investigative tool. The growth of cells in culture, the fusion potential of certain viruses, and the selective effect of specific culture media have made *in vitro* hybridization a reliable and predictable experimental model. Spontaneous random cell fusion and enzymatic cell fusion are not as productive of hybrid cells as viral fusion. However, regardless of the method used in production, the hybrid cells studied were all similar genetically and biochemically. *In vitro* produced hybrid cell lines have been shown to grow *in vivo*. The *in vivo* production of hybrid cells in solid tumors has, however, not been documented.

The present investigation was undertaken to determine whether somatic cell fusion could occur *in vivo* in a solid neoplasm composed of two different neoplastic cell populations. Previously, cells of the murine lines Sarcoma 180 (Foley) and L-5178Y lymphoblast were noted to undergo spontaneous cytoplasmic and nuclear fusion (hybridization) when cultured together *in vitro*. These cell lines were grown as separate and mixed tumors in the thighs of C3H and BDF mice. Growth characteristics of the inoculated tumors were observed over a 21 day period and karyotype analyses were made on cells from the separate and mixed tumors. In the C3H mouse the S-180 grew progressively as a solid tumor, the L-5178Y regressed after the eighth day following inoculation, and the mixed tumor followed an intermediate course. These same tumors all grew progressively in the BDF mouse, as separate and mixed tumors. Hybrid cells were consistently noted in a low frequency (3.1% of tumor cells) in the mixed tumors 21 days following inoculation in the C3H mice.

No hybrid cells were found in the BDF mice. This study demonstrates that the solid tumor state is no barrier to hybrid cell formation. The possibility exists that fusion between clones of cells in a given tumor may occur and provide a mechanism whereby tumor behavior changes.

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INTRODUCTION

Somatic cell hybridization has been established as a means of genetic exchange between cells *in vitro*. This has not been well documented as a mode of behavior of mammalian somatic cells *in vivo*. Malignant somatic cells having the potential of fusing with other somatic cells could give the new "hybrid cell" a selective growth advantage over its parents, because of its new genetic recombination. The demonstration of hybrid cells in a solid mixed tumor establishes that *in vivo* somatic cell fusion is a fact. The viability, the behavior, and the mechanism of producing a hybrid cell *in vivo* remain to be determined.

REVIEW OF THE LITERATURE

I. INTRODUCTION

One of the mysteries of biology is the behavior of the cell and particularly the cancer cell. Medical history records man's search for its secrets. Many theories have been proposed to explain the neoplastic cell's wild disregard for natural regulatory mechanisms. Various etiological and environmental factors have been implicated. Some of the more revelant ones will be reviewed first. More recently, another mode of somatic cell behavior has been discovered. An exchange of genetic material between mammalian somatic cells has been demonstrated *in vitro*. Some of the factors and mechanisms involved in *in vitro* somatic cell hybridization are reminiscent of events seen in carcinogenesis. The second part of the literature review examines these events in light of their possible relationship to cancer cell behavior.

II. CARCINOGENESIS

A. General

Carcinogenesis refers to the cellular changes that lead to the development of a neoplasm. Consideration must be given to etiological factors, as well as to the changes produced in the cell. Initial clinical observations made suggested that repeated trauma to an area, or chronic low grade parasitic infections, were associated with a higher incidence of certain carcinomas and sarcomas. Embryonic rests of fetal tissue, with subsequent mutation or reactivation, have also been implicated. Clenet in 1910 showed x-radiation produced sarcomas in rats. Cellular changes induced by radiation have been shown in

experimental tumors in animals and have also been implicated in human malignancy.[1] More recently, however, the role of carcinogens, with their chronic irritation effect, and viruses, with their mutation producing potential, have kept the attention of researchers. Consequently, our attention will be directed to these areas.

B. Carcinogens

Carcinogens can alter sites within and around the cell which are specific for maintaining normal growth controlling mechanisms. These changes prevent the cell from responding in its usual way to the environment. Changes produced in this way may be heritable.[2,3,4]

Animals have provided most of the evidence for chemical agents acting as carcinogens. Polycyclic hydrocarbons of the anthracene family, certain azo dyes, aromatic amines, and antimetabolites like nitrogen mustard, produce specific neoplasms in experimental rats or mice. Heavy metals like nickel, cobalt, selenium and berylium, also induce malignant cells if imbedded in normal tissue over a long period of time. Burying apparently inert materials, such as cellophane or plastic discs, under the skin, can produce sarcomas in rats and mice.[3,4,5]

Man has not been exempted. The Surgeon General's Report linking cigarette smoking to carcinoma of the lung is well documented with clinical evidence.[6] Cecil Potts in England in 1775 observed a high incidence of scrotal sarcoma in chimney sweeps. Bladder carcinoma has been more common in aniline dye workers.

Only recently have the changes in carcinogenically altered cells been determined. These cellular changes are not only chemically induced, but can also be produced by viruses.

C. Viruses

Viruses, or virus particles, have been cited as possible carcinogenic agents since their discovery.[7,8,9,10] They are known to produce transferable animal tumors by acting through their ability to produce mutogenic changes in cells, or by being directly incorporated into the genetic material of the cell. DNA viruses such as the polyoma, SV40, and adeno virus type 3 are known to produce their effects directly at the nuclear level by combining with the host's DNA itself. Since DNA is a cell regulator, alterations in its structure will be reflected throughout the cell. RNA viruses, of the Rous and Bittner milk variety, exert their influence through the cell cytoplasm.[11] Epigenic action of the virus particle within the cytoplasm have produced permanent cell changes modifying the cell's behavior, but not necessarily its genetic makeup. Although most of the evidence for a viral etiology of malignancy is from animal experimentation, evidence from human lymphomas is accumulating.[12] Both Burkett's lymphoma and, more recently, Hodgkin's lymphoma, have demonstrated virus particles on the neoplastic cell membrane, and specific antibodies produced against these viruses have also been demonstrated.[13,14]

D. Cancer Cell Behavior

i) Gross Characteristics

The cellular response to such influences as viruses, radiation, or chemical carcinogens is the subject under investigation here. Morphologic changes within a neoplastic cell have classically been demonstrated in the nonviable state. Anaplastic or undifferentiated human cells are hyperchromic, variable in size and shape, and have a

greater nucleus to cytoplasm ratio. This cell type has a behavior problem, undergoing mitosis irregularly and more frequently than the normal counterpart; having the potential to migrate and relocate; and having a greater affinity for nutrients, often living at the expense of the normal cells.[15] Extensive and classic reviews of these cancer cell phenomena were published by Coman,[16] Mercer,[17] Abercrombie and Ambrose.[18,19] Specific changes have been demonstrated in the nucleus, cytoplasm, and cell membrane of the tumor cell.

ii) Nuclear Changes

As early as 1912 Boveri showed that the chromosome number in malignant cells varied.[20] Burnette in 1959 postulated that the malignant cell's behavior was a result of mutational changes resulting in extra chromosomes or even loss of certain chromosomes.[21] DeGrouchy is a strong proponent of the chromosomal theory, believing that selective pressures cause the cancer cell karyotype to evolve by acquisitions and losses, or by structural rearrangement so that the cell will eventually outgrow the host.[22] Evidence for such a mode of behavior comes from tissue culture transformations resulting in tumors with malignant tendencies and from the Phi chromosome in chronic myelogenous leukemia.[23,24,25] Other chromosome rearrangements in tumors have been described.[26-31] On the other hand, many neoplasms show no readily apparent change in karyotype and other mechanisms are postulated to account for this phenomenon.[30-33]

iii) Cytoplasmic Changes

Control of cell function by the cytoplasm and cell membrane without alteration of the genetic make-up of the cell nucleus has been demonstrated by many workers.[34-37] Epigenetic mechanisms, working at

the cellular level, may regulate gene function by: (1) turning on or off, or by modifying the production of certain kinds of RNA; (2) regulating cell differentiation by tampering with the translation of RNA into specific enzymes, or (3) by altering the cell membrane protein structure and, therefore, function.[2] Henry Harris has studied this kind of DNA-RNA interaction in artifical hybrid cells of hen erythrocyte and human HeLa cells.[38] This altered biochemistry of a tumor cell is reflected in increased glycolysis and an increase in glycolytic enzymes.[39] Other enzyme changes have been noted.[3,40] Cameron maintains that increased release of hyaluronidase by cell cytoplasm in an attempt to maintain its environment and nutritional requirements, is the first stage in carcinogenesis.[41]

iv) Membrane Changes

Since the cancer cell is different from the normal somatic cell, alteration in recognition of self has been postulated, and now shown to be correct.[2,42,43,44] Antibodies to malignant cell antigens are present in many malignancies. However, the host's response to these tumor antigens is variable because of possible immunological tolerance, a weak tumor antigen or, in certain cases, the antigen may actually give rise to enhancement rather than resistance.[45-46] Viruses that produce antigens in the nucleus, cytoplasm or cell membrane are of two types - the transplantation antigen that behaves in the classic rejection-type phenomenon manner, or a complement fixation tumor antigen that requires activation by complement or other enzyme precursors in order to be recognized by the host. Each viral induced antigen is identical in all tumors produced by that virus, even in different animals or species.[45,46]

Chemical carcinogen produced antigens are also of these two types. However, they are different in all tumors derived from a specific carcinogen even in isogenic animals.[44] The host immune response antibody can be cell mediated as well as through hormonal antibodies.

III. SOMATIC CELL HYBRIDIZATION

A. General

All of the above: nuclear rearrangement, cytoplasmic regulation, and cell membrane alteration, appear to be involved in the recently discovered somatic cell behavior phenomenon of hybridization. Somatic cell fusion is recognized by the nuclear exchange and rearrangement of genetic material (chromosomes). Confirmation of such exchange is seen in the new cell karyotype,[47,48] its production of enzymes related to both parents,[49,50] and the presence of cell membrane antigens from its predecessors.[51] All the etiologic factors determining the hybrid cell's behavior have not been elicited. Viruses and chemical agents have produced hybrid cells *in vitro*.[48,49] *In vivo*, hybridization (when it occurs) presumably can involve these or other factors specific to the host or its environment.

B. History

i) *In vitro* Hybridization

Our understanding of somatic cell hybridization dates back only ten years.[52] The discovery of the process is but one of the specific scientific advances credited to the development of tissue culture techniques. George Barski, Serge Sorieul, and Francine Cornefert at the Institute Gustave Roussy in Paris in 1960, noted that when two murine strains of cells were grown together in tissue culture medium,

a third variety of cell appeared after several months.[53] This cell contained the approximate sum of the two chromosome numbers in each parent line. As well, marker chromosomes from the NCTC 2422 and from the NCTC 2555 were present in the hybrid cell. These hybrid cells survived in culture until a pure line could be isolated. Karyotype analyses during the evolution of the hybrid cell line, showed that chromosomes were selectively discarded so that the final count was lower than the summation of the two parent line chromosome numbers.[54] The term segregation has been used to designate this set of events. Ephrussi in Genetique Physioloquie at Gif France, and later at Western Reserve University, also developed hybrid cell lines by random selection using the same two mouse lines. It is from his earlier work that the segregation idea gained impetus, for he showed that one parent line tends to have a selective advantage over the other in the development of a stable hybrid cell lines.[55,56,57]

The use of selective culture media to induce somatic cell hybridization was employed by J.W. Littlefield from Harvard Medical School in 1964. He developed a pure line of hybrid cells by using two clonal sublines of L cells, each deficient in one enzyme, but who, in combination, could survive as a hybrid in media containing aminopterin. The one parent line, A9, was lacking guanylic acid - enosinic acid pyrophosphorylase, while the other line, B34, was missing thymidine kinase. However, together the resulting cell carried both enzymes necessary to survive with aminopterin in the culture media.[58]

Davidson and Ephrussi modified this technique in what they termed the "half selective" method. In this method only one parent lacked an essential enzyme, but the other parent was slow growing.

This work was done using A9 (which lacked the above mentioned enzyme) and fibroblast-like cells from the secondary culture of skin from newborn CBA mice (the slow-growing cell line). The medium contained hypoxanthene, aminopterin and thymidine which allowed growth of those cells that had complementary metabolic pathways.[59]

So far, only intraspecific hybrids had been demonstrated. Weiss in 1965 developed interspecific hybrids using mouse and rat cell lines.[60] Since then, interspecific hybrids have been produced between mouse-human, mouse-hamster, chicken-human, and chicken-mouse.[61-66] At this time too, Weiss recognized that specific enzymes produced by each cell line could be used to identify hybrids, just as marker chromosomes had been used. Ruddle et al. sophisticated this approach in his hybridization studies.[49,50] Howard Green and M. Weiss in 1967, New York University School of Medicine, went a step further by identifying the specific set of chromosomes that were responsible for thymidine kinase activity in human cell lines. This was possible only because the interspecific mouse-human hybrid showed such a marked preferential loss of human chromosomes. The remaining human chromosomes could be identified as the ones having thymidine kinase activity.[67,68]

<u>In vitro</u> hybridization of somatic cells became more readily reproducible when H. Harris and J.F. Watkins, at Oxford in 1965, produced artificial heterokaryons with cells of two or more nuclei using the virus fusion technique.[63-66] The observation that Sendai viruses (rendered noninfective by ultra-violet light) could produce cell clumping and fusion, is attributed to Y. Okada from Osaka University, Japan.[69] Although many of Harris' heterokaryons did not survive as

cell lines, he demonstrated that the cells with a smaller number of nuclei would undergo mitosis and produce viable daughter cells. It remained for M. Weiss to demonstrate that hybrid cells produced by fusion were capable of multiplication and survival.[68] These hybrids compared favorably with those developed by the selective media technique.[58,70,71] Today a combination of both viral and selective media methods are used to develop hybrid strains for study.

Geneticists have used hybrid cell lines to determine individual chromosome activity. The relationship between nuclear DNA and cytoplasmic RNA has also been studied in the hybrid cell. It is most natural, therefore, to expect that hybrid cells would be used to study the origin of malignant cell behavior. G. Klein and H. Harris reported that hybrid cells produced from a mouse malignant strain, Ehrlich's Ascites Carcinoma (EAC), and a low malignant mouse fibroblast line, A9, failed to develop tumors when implanted in irradiated young mice. Suppression of the malignant tendency seemed to have been demonstrated.[72] This may have been possible by selective chromosome loss, or by indirect suppressive effect of A9 chromosomes. Previous studies have suggested that malignancy remained a dominant feature of the somatic cell hybridization.[48,54,61,73,79]

ii) In vivo Hybridization

In vivo production of pure hybrid cell lines or tumors has proved difficult. Okada first produced in vivo cell fusion of EAC tumor cell lines in 1955 when he injected Sendai viruses into the ascitic tumor suspension in the abdomen of mice and noted the occurrence of binucleated cells in the ascites one hour later.[69] Sigali reports the production of

a solid tumor from a hybrid line in young irradiated mice.[74] However, her original hybrid cells were produced *in vitro* by virus particle fusion using a melanoma and A9 cell lines. The persistence of melanin pigment and of tumor formation was confirmed. Actual production of a hybrid cell *in vivo* may also have been demonstrated by Agnish and Federoff in their work with EAC.[76] Their observation that a large cell with different staining characteristics and a chromosome number equal to the sum of EAC cell and normal mouse lymphocyte, could mean that this cell was a hybrid. No hybrid cells have been demonstrated in solid tumors to date.

A review of certain clinical conditions raises the possibility of somatic cell hybridization occurring as a natural *in vivo* phenomenon. Russell et al., in an extensive review of thyroid carcinoma, point out the presence of follicular and papillary carcinoma loci in thyroid glands consisting mainly of the highly lethal anaplastic thyroid carcinoma.[76,77] Both follicular and papillary carcinomas are relatively slow growing and compatible with co-existence in the host. Could these carcinomas, existing together in a patient, combine to form the anaplastic variety of thyroid carcinoma? Further pathologic evidence of "hybrid cells" comes from the paper of Neville et al. on adrenal cortex tumors.[78] He described, in histologic and biochemical terms, peculiar "hybrid cells" lying between the zona granulosa and zona reticularis cells of the adrenal cortex adenoma and adenocarcinoma. Finally, Sandberg's identification of change in karyotype in the peripheral blood and bone marrow of leukemia patients several weeks prior to an exacerbation of their illness, suggests that a recombination

of malignant cells, or malignant and lymphocyte or macrophage cells, may be responsible for the production of this new cell line.[30] Hybridization of somatic cells as demonstrated *in vitro* must be considered as a mode of behavior of the malignant cell *in vivo*.

C. Methods of Hybridization

In vitro methods that have resulted in nuclear and cytoplasmic fusion of somatic cells are by random contact, the use of selective media, or by active cell fusion using noninfective viral particles or enzymes. Random, but constant, contact between two cell lines was the earliest method used. Cells were cultured together as monolayers, as suspensions, or in a dynamic state (spinner flask). Littlefield's use of selective media and later Ephrussi and Davidson's "half selective" methods, made production of hybrid cells more certain and more consistently reproducible. Growing the two parent lines in media that would gradually eliminate all the cells but those that could synthesize their essential amino acids, as a result of complementary enzymes being in the same cell, provided proof of hybridization. Thus a pure hybrid cell line was produced. Fusion of cells by viral or enzymatic action produces multi-nucleated cells within minutes. This type of cell can be seen to develop within minutes when cells are mixed with HVJ virions, cooled on ice water for 20 minutes, and then incubated at 37°C on a shaker.[79] Viokase (0.25% GIBCO) may be used in place of virions, and after 10 minutes incubation at 37°C, the cells are cooled at 4°C for two hours before cell fusion is prevalent.[49] Obviously, not all fused cells are viable and not all cells are hybrids. Therefore, to eliminate the parent strains, selective media is used to separate the hybrid strains from the

parents. These methods are of an accurate and readily reproducible experimental design so that cytologic, biochemical, and genetic information can be more precisely obtained.

D. Mechanisms Involved in Hybridization

i) Steps Postulated

Advent of the electron microscope and utilization of time-lapse photography have greatly added to our knowledge of cell behavior. Electron microscopy has suggested the mechanism of cell fusion as initiated by the viral incubation technique.[79] These were described by Hosaka and Kashi and can be summarized as follows:

a) Agglutination of cells by the virions in the cold. This is related to the hemoagglutinating ability of the HVJ virions.

b) Close contact of the adjacent cell membranes near areas of adsorbed virions of the agglutinated cells.

c) Degeneration of the cell membranes in contact with the virions and the communication of cytoplasm through the breaks created. This occurs within two minutes after the cells are brought to 37°C. A phagocytic-like response by the cells may have started these last two steps. The lytic activity of the virus capsule has also been implicated in altering the cell membrane at this point.[69]

d) Formation of cytoplasmic bridges by connecting of the broken edges of the cell membranes - also within two minutes.

e) Enlarging of the cytoplasmic bridges and engulfing of the virions as vesicles in the cytoplasm.

f) Rounding up of the fused cells with further alteration of the virions within the vesicles.

The role of the cell membrane cannot be over-emphasized. Alteration of the membrane by the virions lead to breakdown and reforming of the cell wall as bridges, so that cytoplasmic exchange could begin. Rounding up and enlarging of the cell followed. Other requirements for cell fusion are energy,[80] optimal viral concentrations,[81,82] and calcium ions.[83] The exchange of nuclear material is dependent upon mitosis. Binucleate cells have been considered an intermediate cell in hybridization by most authors.[35,48,84,85] Confirmation of the occurrence of mononucleated cells, following cell fusion, suggested two possible methods. Nuclear fusion could take place (1) during interphase, or (2) if both nuclei entered mitosis together. Eighty-five percent of homokaryons that enter mitosis are in synchrony. The mechanisms observed to happen during mitosis are:

a) Production of a single mitotic spindle, a single metaphase plate, and cell division producing two daughter cells, each with a complementary number of parental chromosomes.

b) Tri- and tetrapolar mitosis with cell division producing either a binucleate cell or one with chromosomes from each parent.

c) The cell entering mitosis but failing to divide so that when the chromosomes are gathered at the anaphase stage, either one large nucleus or two nuclei with both sets of chromosomes are produced.[48]

Nuclear rearrangement with the preferential loss of chromosomes of one parent or the other has been termed segregation. Necessity of such a process is determined by environmental factors and the ultimate survival of the new "hybrid" cell. Repeated observations indicate that heterokaryons of more than two nuclei tend not to survive, but cells with chromosomes of two original nuclei can undergo division and become a permanent line.[66]

ii) Co-Factors Involved

Viruses and enzymes have been the main consideration for in vitro cell fusion. Cold, as a clumping factor, and the constant aggitation of media in a spinner flask, have also been implicated.[35] Besides the possibility of viruses and specific enzymes playing a role in in vivo hybridization, other factors are known to cause cell fusion. These include bacteria, parasites, fungi, silica, cellophane, oils, fats, cholesterol and parathyroid hormones. Furthermore, stresses in the form of starvation, radiation damage, or necrobiosis have also produced cell fusion.[86] These factors are known to produce the giant cells seen as part of the immune response of mammals.

iii) Genetic Considerations

Endomitosis is one of the genetic mechanisms that can be confused with hybridization.[87] Until now, some form of endomitosis (endoreduplication) has been suggested for the change in ploidy seen in neoplasia and cell cultures. In this process the cell enters mitosis, the chromosomes are reduplicated, but they fail to separate because of faulty spindle formation. Reduplication during telophase also accounts for production of cells with larger chromosome numbers. Failure of cell division (cytokinesis) has also been demonstrated. In each of these

genetic faults, the resulting cell would likely have a chromosome number twice normal and the chromosomes would be the same as in the original parent cell.[88] Hybrid cells have chromosomes from two parent cells in them.

Loss of chromosomes and internal rearrangement of chromosomes as in crossing-over and nondisjunction, occurs with regularity and is well documented. The fragmentation of chromosomes with subsequent production of bizzare ring or marker chromosomes is also known.[88] These genetic recombinations might be considered responsible for the karyotype of a particular hybrid, however, the regular production of hybrids *in vitro* and the irregular occurrence of these genetic changes in a cell line argues in favor of a mechanism of somatic hybridization.

IV. SUMMARY

1. The behavior of somatic cells, particularly the neoplastic cell has intrigued scientists for several decades.
2. Newer theories of carcinogenesis consider the role played by both chemical carcinogens and viruses in altering the cell's behavior. These carcinogenic agents influence the relationship between the nucleus, cytoplasm and cell membrane.
3. Altered relationships between nucleus, cytoplasm and cell membrane are present in a recently discovered *in vitro* process of somatic cell hybridization.
4. Criteria necessary for somatic hybridization appear to be:
   a) agglutination and close contact of cells,
   b) altered cell membranes,
   c) cytoplasmic-nuclear exchange, and

d) nuclear rearrangement of hybrid cells to remain viable.

5. Clinical examples suggest that the process of somatic hybridization is a function of a somatic cell *in vivo* as well as *in vitro*.

OBJECTIVES

This research work is a part of an experimental study involving mammalian somatic cell hybridization. Initial phases of the work have demonstrated,

a) *in vitro* hybrid cell production from two neoplastic cell lines with distinct karyotypes,

b) that these distinct neoplastic murine lines were transplantable as solid tumors *in vivo*.

Further objectives of this experimental research are

a) to demonstrate *in vivo* hybrid cell production by the above established tumor cell lines,

b) to determine if the *in vivo* hybrid cells contribute to the evolution and progression of the solid tumor state.

MATERIALS

The decision to use mouse cells in the following experiments was made because (a) the L-5178Y and the S-180 (Foley) cell lines were readily available, (b) *in vitro* hybridization had previously been demonstrated between these cell lines, and (c) pilot studies showed that tumors of L-5178Y and S-180 grew in both C3H and BDF mice.

I. L-5178Y MOUSE LYMPHOBLAST CELL LINE

The L-5178Y lymphoblast cell line was obtained from Dr. A.C. Satorelli of Yale University, through the courtesy of Dr. A.R.P. Paterson from McEachern Laboratory, The University of Alberta. This cell line has been maintained *in vivo* in male BDF mice by intraperitoneal transplantation of 1×10^7 tumor cells each week. *In vitro* L-5178Y cells grew in suspension and in spinner flasks in Fischer's Medium for Leukemic Cells, supplemented with 10% horse serum.

II. SARCOMA 180 (FOLEY) CELL LINE (S-180)

The Sarcoma 180 (Foley) cell line was established by G.E. Foley et al.[89,90] in 1959 from an S-180 sarcoma maintained in adult CFW mice. The S-180 (Foley) cells used in this experiment were purchased from Microbiological Associates, Inc.* They normally grew in a monolayer in Minimum Essential Medium supplemented with 10% horse serum. The cells from this line could also be grown in a spinner flask in Fischer's medium with 10% horse serum for four to six weeks.

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* Microbiological Associates, Inc., 4733 Bethesda Avenue, Washington, D.C.

III. MICE

Brown, female, C3H mice ranging in age from two to four months, were used as hosts for the production of tumors from the L-5178Y and the S-180 (Foley) cell lines. Black, male, BDF mice of the same age were also used under the same experimental conditions. These mice were obtained from Microbiological Associates, Inc. They were maintained in cages of ten, and given a standard laboratory murine diet. No infected animals were detected at any time.

METHODS

The cell lines S-180 (Foley) and L-5178Y were maintained *in vitro* to provide cells for an inoculum, as well as to serve as controls in karyotype analysis. Both cell lines were grown as separate tumors and together as a mixed tumor. Animals were killed and tumor pieces were taken at regular intervals for histologic study and for explantation. The explanted tumor cells were prepared for karyotype analysis after 48 hours of incubation at $37^{o}C$.

I. PREPARATION OF CELL SUSPENSIONS

The L-5178Y and S-180 (Foley) were grown separately in Fischer's medium with 10% horse serum in a spinner flask for 48 hours. These cells were then spun down and resuspended in a small aliquot of fresh medium. This suspension was used as the inoculum for the mice. All cell counts were done on a hemocytometer counting chamber. *In vitro* chromosome analyses were also done. Colcemid* (final concentration of 1×10^{-7} mg./cc.) was added to the medium after 48 hours of incubation, for a period of six hours, prior to a sample being taken for karyotype analysis.

II. TREATMENT OF ANIMALS

The C3H and the BDF mice were used in the following groups:

Group A - 10 C3H and 10 BDF mice were given 25×10^{6} L-5178Y cells intramuscularly into the right thigh.

Group B - 10 C3H and 10 BDF mice were given 5×10^{6} S-180 cells intramuscularly into the right thigh.

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* CIBA Company, Dorval, Quebec.

Group C - 10 C3H and 10 BDF mice were given 25 x 10^6 L-5178Y and 5 x 10^6 S-180 cells intramuscularly into the right thigh.

Group D - 10 C3H and 10 BDF mice were given a control intramuscular injection of 0.5 cc. Fischer's medium into the right thigh.

Parts A, B, and C were repeated four times so that a total of 141 C3H mice and 80 BDF mice were studied.

Every Monday, Wednesday and Friday calipers were used to measure the size of the mouse thigh. The greatest tumor diameter was taken as a measure of growth so that tumor growth pattern and subsequent behavior could be determined. The mice were observed for a period of three weeks. A three-week experimental period was chosen because in vitro hybridization had been demonstrated by this time. Furthermore, a pilot study had determined that the size of the inoculum used lent itself to a survival time of approximately three weeks.

III. EXAMINATION OF TUMORS

Each week at least two animals were killed to obtain specimens for eventual karyotype analysis. The tumors were treated in the following manner:

a) A cube of tumor measuring 3 x 5 x 10 mm. was fixed in 10% formalin and stained with H & E for histologic study. Autopsy findings and gross characteristics were also noted.

b) Under sterile conditions, tumor pieces were minced in fresh medium into pieces of less than 1 mm. in size. These were drawn into a sterile capillary pipette and explanted into Layton tubes. Five cubic centimeters of either Fischer's medium with 10% horse serum and

penicillin-streptomycin* or Minimum Essential Medium with 10% horse serum and penicillin-streptomycin was added as the culture medium. Incubation time was 48-72 hours at 37°C. Six to 16 hours prior to treating the explant cell suspensions for chromosome analysis, 0.1 ml. of .001 mg./ml. of Colcemid was added to the explant medium.

c) A suspension of fresh tumor in medium was made, using a tissue homogenator. After filtering, the cloudy suspension was incubated at 37°C for two hours in 15 cc. of either Minimum Essential Medium with 10% horse serum or Fischer's medium with 10% horse serum. Colcemid in a final concentration of 1×10^{-6} mg./ml. was added prior to incubation. Alternately, animals used for preparing the tumor suspension were given 0.2 mg. of Colcemid intraperitoneally four hours prior to killing. In both cases the cell suspension obtained was used for chromosome study.

IV. PREPARATION OF CHROMOSOME SPREADS

Cell suspensions from the spinner culture, the explant cultures, and the tissue homogenate were all treated the same way for obtaining suitable chromosome spreads. Each cell suspension was spun down at 800 rpm for five minutes. The supernatant was decanted, and 10 cc. of a 20% hypotonic solution of Hank's balanced salt solution was added to the cell button. This was left at room temperature for one half hour. One millilitre of a solution of hyaluronidase¶ was added to the test tube. The cells were then resuspended and spun down at 800 rpm for five minutes. After the hypotonic solution was removed, not less than

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* 100 units of penicillin + 100 mcg. of streptomycin per 1 cc. of media.

¶ 0.05 gm. Ovine Testes Hyaluronidase (Sigma Chemical Company, St. Louis, Missouri) in sodium hypophosphate - citric acid buffer, pH 5.3.

five cubic centimeters of freshly prepared fixative of (3:1) methanol: glacial acetic acid was added to the cell button. This preparation was chilled for at least one half hour at $4^{o}C$. The cells were resuspended in the fixative, spun down at 800 rpm for five minutes. After decanting, new fixative was added to the cell button for final resuspension. A capillary pipette was used to drop the cell suspension onto a moist glass slide chilled in absolute methanol. The slides were flamed and, after drying, were stained in orecin acetate and cresyl violet. Karyotype analyses were carried out from these preparations.

RESULTS

I. CELL SUSPENSIONS

The L-5178Y grew vigorously in a spinner culture. From an inoculum of 3×10^6 cells, approximately 3×10^7 cells could be expected within 48 hours. The generation time of the L-5178Y was 9.1 hours.* Chromosome analysis of the L-5178Y cell line indicated a bimodal distribution of the chromosome number. In 90 of 100 cells counted, the modal number was 42 (s), and in 10 of the 100 cells, the chromosome number was close to the double stemline number (2s) of chromosomes (Tables I, II and Fig. 1). There were no marker chromosomes present. The karyotype of the L-5178Y was composed of acrocentric and telocentric chromosomes (Fig. 3).

The S-180 (Foley) cell line was suitable for short term culture in Fischer's medium in a spinner flask. From an inoculum of 3×10^6 cells, one could expect 9×10^6 cells within 48 hours. The generation time was calculated to be 18.6 hours. Chromosome analysis of the S-180 cell line demonstrated a range in chromosome number from 61 to 86 with a modal number of 80 chromosomes (s). The double stemline number (2s) of chromosomes was found twice in 100 cells (Table I, II and Fig. 2). The karyotype was composed of acrocentric and telocentric chromosomes, except for three distinctive metacentric chromosomes (one large, one

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* $$tg = \frac{t}{3.3 \log \left(\frac{N}{N_o}\right)}$$

where tg = generation time; t = time in the exponential growth phase; N = number of cells at t; N_o = number of cells initially.

medium and one small) and, on the average, two minute chromosomes (Fig. 4).

II. TUMOR STUDIES

A. Growth Curves

i) L-5178Y Tumor

C3H Mice: The large inoculum of 25 x 10^6 cells was used to insure 100% take. A pilot study had shown that the tumor could be grown in C3H mice if a large enough inoculum was used. The tumor developed slowly, and reached a maximum average size of 0.88 cm. in seven days. The tumor was uniformly rejected in 21 days (Table III, Fig. 5).

BDF Mice: The BDF mice were natural hosts for the L-5178Y cell line. Growth as a solid tumor in the thigh progressed rapidly and ascitic fluid formed by the fifth day. An early death and the presence of ascites were constant features of this tumor. On the fourteenth day, the tumor size was 2.23 cm. and the first animals were dead (Table IV, Fig. 6).

ii) S-180 (Foley) Tumor

C3H Mice: The inoculum of 5 x 10^6 cells resulted in a 100% take of the tumor, and an average survival time of at least 21 days. The tumor increased rapidly in size and showed continual growth to the time of the animal's death. Although there was central necrosis, the tumor showed a progressive increase in size throughout its duration. An average diameter of 2.61 cm. was reached at 21 days. The animals became emaciated at the time of death (Table III, Fig. 5).

BDF Mice: Animals with 5 x 10^6 cells of S-180 inoculated into the right thigh all developed tumors that continued to grow throughout

the life of the animal. The tumor grew to a larger diameter than the L-5178Y. It showed a progressive growth pattern and developed into tumors that averaged 2.82 cm. by 21 days (Table IV, Fig. 6).

iii) Mixed Tumor

C3H Mice: The complementary number of 25 x 10^6 L-5178Y cells and 5 x 10^6 S-180 (Foley) cells was used as inoculum. The tumor produced by this inoculum showed an intermediate growth pattern to that of L-5178Y and the S-180 (Foley) tumors. Although there was central necrosis in the tumor, this fact alone does not account for the unexpected resolution of tumor size toward the end of the three week growth period. Loss of the L-5178Y mass would account for some of the change in leg diameter, but regression of the tumor seemed unlikely in view of the previously demonstrated virulent nature of the S-180 (Foley). The growth curves for the C3H mice are shown graphically in Fig. 5. Table III gives the mean diameter and standard deviation of tumor growth during the three week period.

BDF Mice: The growth pattern in the BDF mice showed the summation effect of the two tumors. The full morphological expression of both tumors was shown. Persistent growth throughout the life of the animal was a characteristic inherited from the S-180. Ascites and an early death, characteristics of the L-5178Y, were also found in the mixed tumor. Table IV gives the mean diameter and standard deviation of the tumor growth during the three week growth period in the BDF mice. Fig. 6 shows the growth curves during this time.

B. Gross Pathology and Histology

i) L-5178Y Tumor (Fig. 7)

The L-5178Y tumor in the C3H mice was a pinkish white color on

the cut section. Areas of necrosis and caseation were frequent. On histologic section, small undifferentiated lymphoblast cells were scattered throughout the muscle fibres. Later other round cells were present and necrotic debris formed in the tumor implant. No metastases or ascites developed.

In the BDF mouse, the tumor was off-white in color and showed a homogeneous nature. There were central areas of necrosis present. Occasionally, liver metastases were noted. Hemotogenous spread at the time of injection could explain these metastases. Ascites was present in all the mice. In addition, subcutaneous edema fluid containing tumor cells was present over the abdomen. L-5178Y cells could be grown from both the ascitic and edema fluid. Histologically, the tumor consisted of loose sheets of small, round, undifferentiated lymphoblast cells.

ii) S-180 (Foley) Tumor (Fig. 8)

In both C3H and the BDF mice the gross and histologic features of the S-180 were similar. Grossly, the tumor was a fleshy pink color with areas of necrosis. No metastases were noted. The animals died by local extension of the tumor and of gross malnutrition. Histologically, a sarcomatous pattern of undifferentiated cells was seen, suggestive of the fibroblast origin of the S-180. An increased nucleus-to-cytoplasm ratio was evident and mitotic figures could be seen scattered throughout the histologic section.

iii)Mixed Tumors (Fig. 9,10,11,12)

In the C3H mouse the mixed tumor was smaller than the S-180 but resembled it on gross section. The central areas of necrosis and

caseation were surrounded by fleshy pink tumor tissue. No metastases were demonstrated in the liver or lungs. The tumor tended to extend locally. On histologic section, the S-180 cells were scattered throughout the clumps of lymphoblast cells. Isolated whirls of large vesiculated cells were found. Binucleate cells were present in these areas. These may represent hybrid cell precursors.

The BDF mixed tumors consisted of a mixture of pink and white tissue with areas of necrosis scattered throughout the tumor. Local lymph nodes were slightly enlarged and ascites was present. On histologic examination, both parent cell lines of the L-5178Y and S-180 could be identified at one week. The L-5178Y dominated the picture at the three week interval. The overall picture of the tumor was still one of undifferentiated cells.

iv) Control

The injection sites in the C3H and BDF were difficult to find and consisted of a few scattered cells of the granulocyte and mononuclear cell lines about a central area of necrosis.

C. Karyotype Analyses

i) Layton Tube Explants

The method of explantation used proved satisfactory. Obtaining good chromosome spreads from these explants was, however, difficult and only 15% of the chromosome spreads were useful for detailed study. Of these spreads, positive identification of each parent line was essential to the accurate counting of the chromosomes present. Explantation in Minimum Essential Medium with 10% horse serum proved to be unsatisfactory because of slow growth and generally poor yield of

satisfactory chromosome spreads. Fischer's medium with 10% horse serum was used routinely for the remainder of the explantations.

a) L-5178Y Tumor

C3H Mice: The L-5178Y was rejected by the C3H mice starting after eight days, therefore no explants of L-5178Y grew after the first week following inoculation. Each intact cell with chromosomes suitably spread was examined under high power and/or oil emersion. Chromosome number ranged from 35-45 with a mode of 40. There were no marker chromosomes. Three cells of 100 fell into the double stemline number (2s) of 80, as can be seen in Fig. 1 and Table I. These show the *in vitro* and *in vivo* chromosome numbers of L-5178Y in the C3H mice.

BDF Mice: L-5178Y lymphoblast cells were maintained as ascites tumor in the BDF mice. Explants of the L-5178Y as a solid tumor grew rapidly and showed a chromosome range of 32-45 with a mode of 40. Eight cells of 100 were in the 2s range. No marker chromosomes were present. The acrocentric chromosomes seen resembled those of the normal mouse. Table II gives the range and mode of the L-5178Y tumor chromosome number in the BDF mice.

b) S-180 (Foley) Tumor

C3H Mice: Growth from the S-180 tumor explant was slower than from the L-5178Y. This was probably a reflection of its generation time and its adaptability from the solid state to a suspension in Fischer's medium. Chromosome number showed a range from 70-86 with a mode of 80 (Table I). There was one double S-180 seen in each 100 spreads. Three metacentric chromosomes identified the spread as an S-180 tumor cell. Figure 2 shows the *in vivo* and *in vitro* chromosome numbers of the

S-180 in the C3H mice.

BDF Mice: Table II gives the chromosome number, range, and mode of the S-180 cell grown in the BDF mouse. The range in chromosome number was 68-84 with a mode of 80. Identification of the S-180 was on the basis of the three metacentric chromosomes.

c) Mixed Tumor

C3H Mice: Both cell lines S-180 and L-5178Y grew from mixed tumor explants. Hybrid cells in a low percentage were identified in the mixed tumor. The number of each cell line and the number of hybrids are given in Table V. Figure 13 graphically illustrates the results of 800 cells counted from a mixed tumor. Of these, 3.1% were hybrids, 78.8% S-180, and 18.1% L-5178Y. Hybrid cells were identified on the basis of the three metacentric chromosomes and a chromosome number of 115 to 150. Figures 14 and 15 show idiograms of a hybrid cell. Figure 16 shows an intact hybrid cell. It should be noted that one hybrid cell showed four metacentric chromosomes. The presence of four metacentric chromosomes in an S-180 had been documented in previous work in this laboratory. It is of interest that hybrid cells could be identified where the number of S-180 cells in the mixed tumor was dominant. Where L-5178Y cells completely dominated the tumor, as evidenced by explantation, no hybrids were found. The presence of L-5178Y cells at three weeks in an explant of a C3H mixed tumor was interesting, since L-5178Y cells failed to grow as a pure line in C3H mice after the first week.

BDF Mice: L-5178Y cells dominated the mixed tumor in the BDF mice. No S-180 cells could be identified in our explant preparations nor were any hybrid cells present (Table VI). The overgrowth of L-5178Y was

likely due to the size of the inoculum into a natural host and the ease of explantation of L-5178Y cells into Fischer's medium.

ii) Tumor Suspension Results

Preparation of chromosome spreads directly from the mixed tumor using a tumor homogenator, was abandoned after the first few weeks, because of (1) poor growth of cells in the suspension, (2) large amounts of debris present, (3) poor yield of adequate chromosome spreads, and (4) the success of the explant method as described above. Treatment of the animal with Colcemid prior to obtaining a tumor specimen for homogenation was not as an effective a way of stopping the cells in metaphase as was treating the tumor homogenate directly. The results of counts done on a mixed tumor homogenate showed that the mixed tumor had 92.2% S-180, 3.4% L-5178Y and 4.4% hybrid cells in metaphase at three weeks (295 cells counted).

DISCUSSION

In vivo somatic cell fusion in solid tumors has been demonstrated between the murine cell lines S-180 (Foley) and L-5178Y. Chromosome analysis was used to identify hybrid cells from the mixed solid tumor state. Criticism and support of the method used forms the first part of the discussion. The second part of the discussion is an interpretation of the results obtained.

I. CRITICISMS AND SUPPORT OF THE METHOD

A. Reproducibility and Reliability of Method

Growth of the solid tumors in mice was reproducible within the limits shown in the tables of mean diameter and standard deviation (Tables III, IV). Some of the variables that affected tumor size and growth were age of the mice at the time of inoculation, quantity of inoculum given, and thigh diameter at the site of the inoculum. It is felt that an acceptable degree of tumor uniformity was obtained.

Explantation of tumor pieces into culture media had inherent difficulties in it that could influence its reliability. The age of the tumor, the site of biopsy of the tumor piece, and the size of the biopsy are the difficulties related to the technique of explantation. Another difficulty, namely, tumor adaptability to culture media, most obviously affected the yield of cells suitable for chromosome analysis. L-5178Y cells normally propagate indefinitely in Fischer's medium. S-180 cells are maintained indefinitely as a monolayer in Minimum Essential Medium, but propagate in a spinner flask with Fischer's medium for periods of four to six weeks only. Both Fischer's medium and Minimum Essential

Medium were used in attempting to grow hybrid cells from tumor explants. Fischer's medium proved the most reliable for culturing hybrid cells found in the solid mixed tumors. The generation times of the two cell lines differed considerably. These times were 9.1 hours for the L-5178Y and 18.6 hours for the S-180. Differences in generation time probably accounted in part for the number of each cell line grown from the mixed tumor. Mitotic indices varied from preparation to preparation but also reflected the tumor's adapability to the medium, and obviously determined the number of cells available for counting.

B. Explant Incubation Period

Could the hybrid cells identified be the result of an *in vitro* fusion during the 48 hour period of incubation of the tumor pieces? The likely answer to this question is no. *In vitro* hybridization between the L-5178Y and S-180 (Foley) occurred randomly only after 21 days in spinner culture. Forty-eight hours in a Layton tube provided only enough time for growth of two or three cell generations, after the latent period of adaptation to the medium. It, therefore, seemed unlikely that hybrids could be formed in the explantation growth period. Explantation of binucleate tumor cells from the mixed tumor, that would then go on to form recognizable hybrid cells in the explantation medium, does not detract from the idea of *in vivo* hybridization.

Lubs and Kotler have shown that the karyotype of the solid tumor does not vary for three days (generations) after explantation.[92] Parshad and Sanford have confirmed this in their work on the effect of different media on tumor explantation. They found that there was no variation in karyotype even as late as nine days.[23] The *in vitro* and

in vivo chromosome number and mode are similar for both the L-5178Y and S-180 (Fig. 1,2). It is reasonable, therefore, to assume that the cells explanted from the solid mixed tumor are representative of that tumor, including the hybrid cells identified.

Attempts to obtain cells suitable for chromosome study directly from the mixed solid tumors without explantation, proved generally unsatisfactory. However, several chromosome preparations obtained directly from the solid mixed tumor showed the presence of hybrid cells in a low percentage (4.4%). Obtaining these hybrid cells directly from a mixed tumor suspension underscores the authenticity of the hybrid cells recovered consistantly in the explantation studies.

A further possibility is that the incubation time of 48 hours was too short for newly formed *in vitro* hybrid cells to reach metaphase. Roa in his studies on DNA synthesis and mitosis in hybrid cells, demonstrated that the nuclei following cell fusion wait for the one with the longer generation time to finish DNA synthesis before going into mitosis together.[93] Thus one would expect a long initial growing period before newly formed hybrid cells would go into mitosis. If hybrid cells formed *in vitro* in the explant media, they could likely have been missed in a 48 hour incubation period.

Growth rates of established hybrid lines *in vitro* are variable. Hybrid lines with characteristic "hybrid vigor" often outgrow their parents and have shorter generation times. Generation times equal to, and intermediate to, that of their parents are frequent. Also, heterokaryons such as the HeLa - Ehrlich ascites tumor mating have a much longer life cycle than either of the parents. Fusion of the same parent

lines may result in hybrids with varying growth rates.[48] With this knowledge in mind, it seems reasonable to consider that the hybrid cells in the solid tumor at three weeks had established a growth rate and were viable and, therefore, short term explantation of the tumor would allow the appearance of hybrid cells in a chromosome analysis preparation.

The treatment of cells with Colcemid stopped dividing cells in metaphase. Stubblefield et al. and others demonstrated that the toxic effect of Colcemid can induce chromosome variations such as bizarre shapes, cell fusion, and pulverization.[94,95,96] These changes were dependent upon concentration and length of time Colcemid was in the culture. Chromosome pulverization and unwinding and shortening of chromosomes, was seen in some earlier preparations of this experiment. Although the Colcemid concentration used was optimal, the length of time in culture was nearing the generation time of the S-180 (18.6 hours). Fusion of cells (actually lack of cell division) could have occurred if the length of Colcemid treatment was such that cells escaping the first block, could enter mitosis a second time.[94] Shortening of the treatment time to six hours did not change the consistency or incidence of hybrid cells found. Therefore, the cells identified as hybrid cells were not the result of Colcemid treatment.

Harris suggests that if the nuclei of heterokaryons are not in synchrony, the incidence of mitosis goes down and abnormal mitosis occurs. No spindle formation takes place, and extensive disruption of chromosomes in the lagging nuclei leads to pulverization or bizarre microchromosomes.[48] This may have lead to some of the chromosome abnormalities seen in this research work. Fig. 14 and 15 demonstrate

some of the unusual chromosomes seen in hybrid cells taken from the solid mixed tumor.

II. INTERPRETATION OF RESULTS

A. Hybrid Cells

Somatic cell hybrids *in vitro* were initially identified by chromosome analysis. Chromosome analysis was also used to identify *in vivo* hybrid cells. The hybrid cells identified were probably the result of the 1:1 combination of S-180 and L-5178Y. Hybrids with a total chromosome number of about 150 may represent the combination of s S-180 with a 2s L-5178Y. Selective loss of chromosomes following cell fusion (segregation) would account for the lower number of 150 chromosomes rather than the number of 160 chromosomes that would be expected from the initial cell fusion. Other genetic mechanisms of endomitosis and nondisjunction of the chromosomes at metaphase have been suggested to explain hybrid cells. Endomitosis would produce a tetraploid cell in which one would expect to see 160 chromosomes and six markers in the case of S-180, and 80 or 160 acrocentric chromosomes in the case of L-5178Y. Nondisjunction could account for smaller increases in chromosome number but not for the production of marker chromosomes. A recombination of chromosomes by a mechanism of somatic cell fusion best explains the hybrid cells found in the solid mixed tumors of C3H mice.

The incidence of hybrid cells in solid mixed tumors was 3.1%. This is compared with the 4.5% incidence obtained *in vitro*. Statistically this difference was not significant ($X^2 = 0.9208$, $P = 0.50 - 0.95$). Viable hybrid cells were found after three weeks of growth in spinner

culture. In the live animals, no hybrid cells were found in the solid tumor before three weeks. This evidence suggests that spontaneous cell fusion with production of viable hybrid cells requires three weeks using the L-5178Y and S-180 cell lines.

It was obvious that the low incidence of hybrid cells could not account for the intermediate behavior of the mixed tumor. Furthermore, no changes in tumorogenicity as expressed by metastasis, more rapid debilitation of the host, or predominant changes in the gross or histologic appearance of the tumor were associated with the solid mixed tumor in the C3H mouse.

B. Tumor Growth Characteristics

i) C3H Mice (Fig. 5)

S-180 cells have been grown in tissue culture for over ten years. During that time they have lost most of their original CFW mouse specificity. Consequently, the S-180 cells grow in a progressive fashion in most strains of mice. Even rats given a high inoculum of S-180 cells will develop solid tumors. The progressive growth pattern of the S-180 solid tumor in C3H mice was, therefore, not unexpected.

L-5178Y cells grew progressively in BDF mice as an ascites tumor, but appeared to be antigenically foreign to the C3H mice. These lymphoblast cells may have maintained a high degree of host specificity and, therefore, behaved as an allograft when inoculated into the C3H mice. The regression of the L-5178Y solid tumor in the C3H mice followed the usual course of a first set rejection. Rechallenging the mice with L-5178Y cells did not produce any further tumor formation. BDF mouse skin grafting of C3H mice challenged with L-5178Y cells, however,

showed a first set rejection of the skin graft. This supported the concept that the tumor regression was not on the basis of a BDF-C3H histo-incompatibility, but rather on the basis of rejection of a specific antigen related to the lymphoblast element of the L-5178Y cell. Further studies are being done to determine more precisely what the immune response of the C3H mice has been.*

The intermediate growth of the mixed tumor in the C3H mice was explained in part by the rejection of the L-5178Y cells. Unexpected, however, was the persistence of L-5178Y cells in the mixed tumor along with the gradual disappearance of the S-180 cells. This was witnessed in both the histology sections and in the tumor explants. When the S-180 cells showed a higher survival rate, hybrid cells were found. These results could be explained on the basis of tumor sampling, tumor adaptability to culture medium, and the different generation times of the L-5178Y and S-180 cells. The persistence of the L-5178Y in the mixed tumor is still problematic when it is recognized that L-5178Y cells were not recoverable from the C3H mice after the first week of growth as a pure tumor. The host response, both immunological and nutritional, must be considered to play a role here. Challenging the animal with a large number of L-5178Y and S-180 cells could have overwhelmed the immunologic response of the C3H mouse, permitting a longer survival time of the L-5178Y (tolerance). Another possibility is that the growing L-5178Y cells successfully isolated clumps of S-180 cells from a nutritional supply, leaving the S-180 cells to die in a battlefield of macrophages, lymphocytes, and L-5178Y cells. Histology sections

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* Unpublished results, courtesy of Dr. W.D. Armstrong, Department of Pathology, The University of Alberta.

of the mixed tumors at one, two and three weeks suggests a change in the character of the S-180 cells (Fig. 7,8,9,10,11). The appearance of larger, vesicular, fibroblast-like cells and the appearance of multi- and binucleate cells by three weeks of tumor growth may well represent an area of hybrid cell formation (Fig. 12). No such areas were seen in the pure parent cell lines S-180 and L-5178Y. The induction of a stromal response by the S-180 and L-5178Y could also account for the appearance of an apparent cell change in the fibroblast line.

ii) BDF Mice (Fig. 6)

Both L-5178Y and S-180 grew progressively in the BDF mice as separate and mixed tumors. A standardized size of the mixed inoculum (25×10^6 cells of L-5178Y, 5×10^6 cells of S-180) produced earlier death in the BDF mice than in the C3H. It was therefore necessary to sample the tumors at a five day interval rather than weekly. The summation effect of two rapidly growing tumors was demonstrated by the mixed tumor group. Early work by Graff et al. and Migliarese et al., showed such an effect might be expected when two established tumors were grown together in the same animal.[97,98]

Histologic sections confirmed a predominance of L-5178Y cells in the mixed tumor. This was probably a reflection of the size of the inoculum. No hybrid cells were found in the BDF mice under these experimental conditions. Variation of the ratio and size of the inoculum might well provide a better environment for somatic cell hybridization in the BDF mice.

C. Possible Factors Involved in Somatic Cell Hybridization In vivo.

Consideration has been given to the production of hybrid cells

from a fusion of one L-5178Y with one S-180. This combination was favoured on the basis of the observed chromosome arrangement. An intimate degree of contact is required for the first step in cell fusion. Electron microscopy has demonstrated a greater number of microvilli and cell membrane irregularities on malignant cells than on normal somatic cells.[43] These features provide greater potential areas of contact between malignant cells rather than between malignant and normal cells. However, the possibility still exists that normal cells may participate in cell fusion. Chromosomes from normal somatic mouse cells closely resemble the L-5178Y cells. Since identification of hybrid cells was dependent on identification of marker chromosomes from the S-180, the other acrocentric chromosomes could have come from normal cells. Cells likely to undergo fusion with S-180 might be from the macrophage series or possibly from the connective tissue cell lines. Although Agnish and Federoff suggested lymphocyte fusion with EAC cells in an ascitic medium, several factors appeared to be against this combination. Firstly, the lymphocyte or macrophage is a differentiated cell and would have to be induced into mitosis by the malignant cell. Chambers and Weiser demonstrated that the action of host macrophages on tumor cells was lytic in nature, therefore not likely to be involved in cell fusion.[99] Okada in his review of cell fusion states that lymphocytes and polymorphonuclear cells were not fused in suspension with HVJ (Sendai virus).[69] Despite these arguments against the involvement of lymphocytes or macrophages in cell fusion, recent <u>in vitro</u> evidence still favors this possibility. Harris stated that fibroblasts, macrophages, and lymphocytes undergo fusion with themselves as well as with other cells under specific conditions, using the Sendai virus.[48,66,100]

Siniscolo presented evidence that in intraspecific fusion, differentiation was not a barrier to viable cell fusion.[100] Harris supported this conclusion in his model of cell fusion with hen erythrocytes and HeLa cells. Reactivation of the hen erythrocyte nucleus was dependent upon the metabolic activity of the cells fused. The HeLa cytoplasmic signals to the hen erythrocyte nucleus induced DNA and RNA synthesis in this inactive nucleus. Differentiation was, therefore, shown to be reversible.[48] Connective tissue cells of mesenchymal origin induced by the S-180 might have been present in the mixed tumor. These fibroblasts could have been involved in hybrid cell production. *In vitro* hybridization of normal somatic cells with malignant cells has been demonstrated.[66,70,73,74,79] However, in the present study, no hybrid cells were found in either of the pure parent solid tumors. Random fusion of normal cells with a malignant cell was not demonstrated in this experimental model.

Etiologic factors that could have induced hybrid cell formation *in vivo* fall into three main groups. These are viral participation, host response mechanisms, and a product of inoculum size and cell contact time. Virus contamination of the cell lines L-5178Y and S-180 prior to inoculation may have occurred, but evidence for or against this event was lacking. Most mouse leukemias seem to have been viral induced, so the presence of viral particles in the L-5178Y remains a possibility.[12] Other viruses participating in *in vivo* hybridization may be considered, although this is less likely to have happened. Nevertheless, even should any of the above situations have been present, the validity of somatic cell hybridization *in vivo* cannot be denied.

The role of the host in response to neoplastic cell challenge is only partially understood. Stress, nutritional factors, and biochemical reactions at the cellular level may play a role in cell fusion. Roizman, in his review of polykaryocytosis, stated that free lipids and lipophilic substances cause alterations in cell membranes leading to giant cell formation.[86] Furthermore, since hybrid cells were found only in the C3H mice which rejected the L-5178Y, the host immune response as an etiologic factor in *in vivo* cell fusion must be considered. Determining the role of the antibody-antigen reaction in the C3H experimental model deserves further study.

Finally, the failure to demonstrate hybrid cells in the BDF mouse does not mean somatic cell hybridization did not or could not happen in that animal. Varying the size and ratio of the inoculum to provide more exposure time for intimate contact between the L-5178Y and S-180 cells might be all that was required to produce viable hybrid cells.

D. *Significance of In vivo Hybridization*

The most significant implication of the demonstration of hybrid cells in solid tumor form is that somatic cell hybridization *in vivo* must be considered as a mode of neoplastic cell behavior. Evolution of a new cell line with a change in tumorogenicity might be an obvious benefit of such a genetic rearrangement. Clearly not all hybrid cells produced would be viable, nor would they necessarily immediately alter the character of the specific tumor. However, it is known that there is a greater variation in chromosome number of a neoplastic stemline, which may give it a selective advantage over the host cells. Reference

can again be made to the phenomenon in human malignancies. The well differentiated (i.e. papillary and follicular) thyroid carcinomas can apparently evolve into an anaplastic carcinoma.[76] The finding of giant cells in the anaplastic variety of thyroid carcinoma only adds substance to the proposed evolution.

Demonstrating hybrid cells in a solid mixed tumor raises the possibility that *in vivo* hybridization may be a useful tool in identifying certain cell characteristics of the parent lines. The metastatic behavior of a tumor may be related to a specific chromosome. Rearrangement of chromosomes in a hybrid cell might produce the loss of that specific chromosome and, subsequently, the inability of the hybrid cell to produce metastases. Similarly, alterations in the metabolic and antigenic behavior of the involved cell lines could be studied using hybridization as an investigative tool.

E. Future Studies

Further studies of *in vivo* hybridization must include an attempt to identify specific inducing co-factors leading to cell fusion. The role of the host immune response to cell hybridization sounds particularly attractive. Determining which cells actually take part in *in vivo* hybridization is important. Although fusion of malignant cells with each other remains the first probability, the participation of the macrophage, lymphocyte, or fibroblast cells in hybridization challenges basic concepts. The finding of the specific cell fusion inducers and the specific cells involved in *in vivo* cell fusion would make *in vivo* somatic cell hybridization a more attractive biological investigative tool.

Predicting the probability and frequency of cell fusion in certain situations could make hybridization an attractive means for constructive manipulation. Harris' *in vitro* work of the apparent suppression of malignancy in a heterokaryon of EAC and mouse fibroblast lines serves as an example of manipulation by hybridization.[72] Defendi also demonstrated that when undifferentiated tumor cells were fused with myoblasts undergoing differentiation into muscle syncytia *in vitro*, the tumor cells did not inhibit the differentiation, but were incorporated into the myotubules of the muscle tissue.[101]

SUMMARY AND CONCLUSIONS

1. In vivo somatic cell hybridization has been demonstrated to occur in the solid mixed tumor of the L-5178Y and S-180 cell lines in the C3H mouse.

2. The hybrid cells produced did not contribute significantly to the tumorogenicity of the solid mixed tumor.

3. Somatic cell hybridization must be considered as a means of neoplastic cell behavior in vivo. Other cells of the macrophage, lymphocyte, and connective tissue cell lines may well be induced to participate in this process.

4. Further studies of this experimental model could be rewarding. Attention is drawn to the question of the role of the immune response in somatic cell fusion.

TABLE I

CHROMOSOME RANGE AND MODE IN VITRO AND IN VIVO IN C3H MICE

	S-180		L-5178Y	
	Range	Mode	Range	Mode
In vitro (100 cells counted)	61-86 (2 cells in the double S-180 range)	80	38-44 (10 cells in the double L-5178Y range)	42
In vivo (100 cells counted)	70-86 (1 cell in the double S-180 range)	80	35-45 (3 cells in the double L-5178Y range)	40

TABLE II

CHROMOSOME RANGE AND MODE IN VITRO AND IN VIVO IN BDF MICE

	S-180		L-5178Y	
	Range	Mode	Range	Mode
In vitro (100 cells counted)	61-86 (2 cells in the double S-180 range)	80	38-44 (10 cells in the double L-5178Y range)	42
In vivo (100 cells counted)	68-84 (2 cells in the double S-180 range)	80	32-45 (8 cells in the double L-5178Y range)	40

TABLE III

GROWTH OF S-180, L-5178Y AND MIXED SOLID TUMORS IN C3H MICE

Day of Tumor Growth	L-5178Y - 47 mice		Mixed - 48 mice		S-180 - 36 mice	
	Mean Diam. (cm.)	S.D.	Mean Diam. (cm.)	S.D.	Mean Diam. (cm.)	S.D.
1						
2			0.62	0.03	0.60	
3						
4	0.55	0.12	0.84	0.16	0.81	0.11
5	0.62	0.05	0.84	0.11	0.94	0.08
6	0.65	0.20	1.48	0.08	1.30	0.32
7	0.88	0.31			1.37	0.32
8			1.63	0.13	1.61	0.29
9	0.77	0.35	1.08	0.50		
10	0.84	0.09				
11	0.54	0.18	1.09	0.38	1.81	0.15
12						
13			0.83	0.31	2.00	0.16
14	0.56	0.05	0.78	0.40		
15			0.76	0.23	2.17	0.14
16	0.52	0.04	0.71	0.37		
17			0.59	0.07		
18	Not				2.50	0.25
19	Palpable		0.58	0.08		
20						
21					2.61	0.33

TABLE IV

GROWTH OF S-180, L-5178Y AND MIXED SOLID TUMORS IN BDF MICE

Day of Tumor Growth	L-5178Y - 21 mice		Mixed - 28 mice		S-180 - 21 mice	
	Mean Diam. (cm.)	S.D.	Mean Diam. (cm.)	S.D.	Mean Diam. (cm.)	S.D.
1						
2	0.52	0.08	0.72	0.09	0.69	0.09
3						
4						
5	0.93	0.30	1.20	0.14	1.02	0.18
6						
7	1.58	0.09	1.67	0.09	1.51	0.05
8						
9	1.80	0.14	1.78	0.10	1.66	0.08
10						
11	2.05	0.21				
12	2.28	0.11	2.09	0.25	1.88	0.08
13						
14	2.23	0.24	2.25	0.13	2.20	0.39
15						
16	2.50	0.20	2.7(1)		2.32	0.22
17	all dead		all dead			
18						
19					2.78	0.35
20						
21					2.82	0.26

TABLE V

CELL TYPES IN MIXED SOLID TUMORS IN C3H MICE

Day of Tumor Growth	L-5178Y %	S-180 %	Hybrid Cells %
7	98.0	2.0	0
14	98.0	2.0	0
21*	18.1	78.8	3.1

* 800 cells counted

TABLE VI

CELL TYPES IN MIXED SOLID TUMORS IN BDF MICE

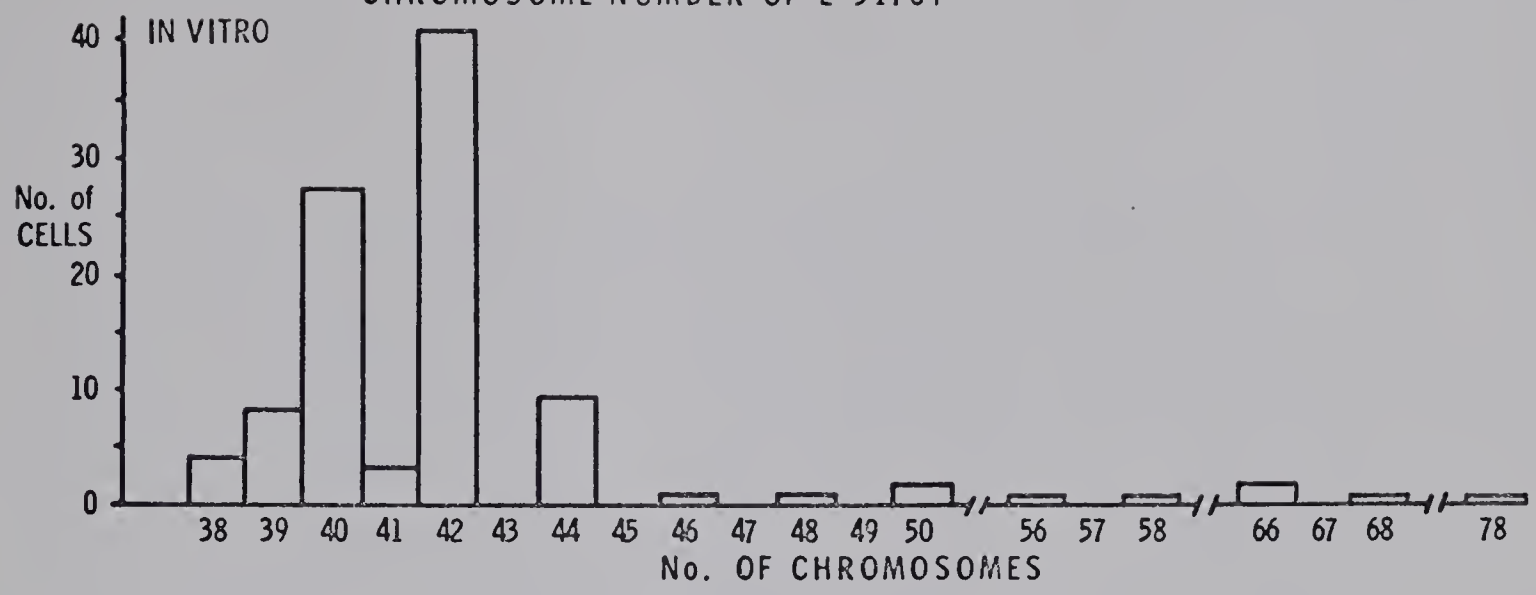
Day of Tumor Growth	L-5178Y %	S-180 %	Hybrid Cells %
5	100	0	0
10	100	0	0
15	100	0	0

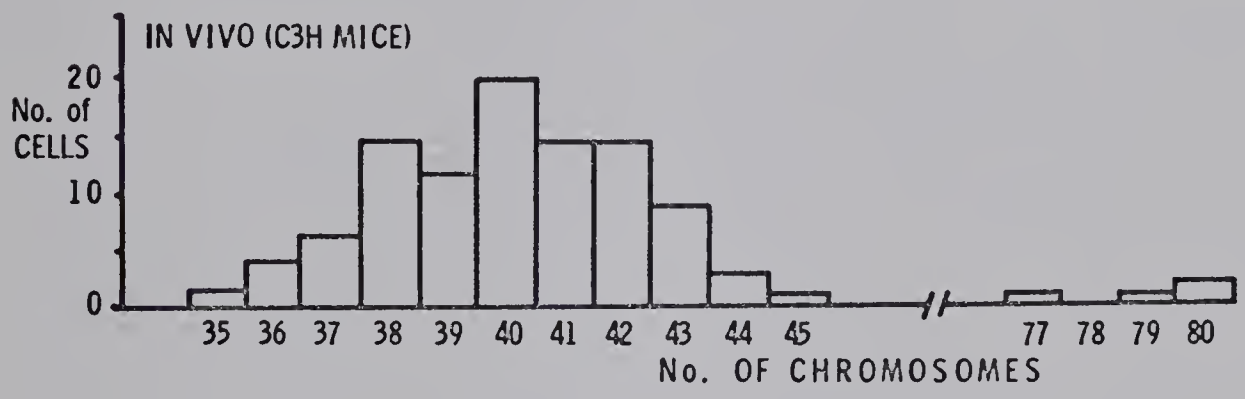
100 cells counted

Fig. 1 Graphs illustrating chromosome number and range of L-5178Y *in vitro* and *in vivo* in the C3H mouse.

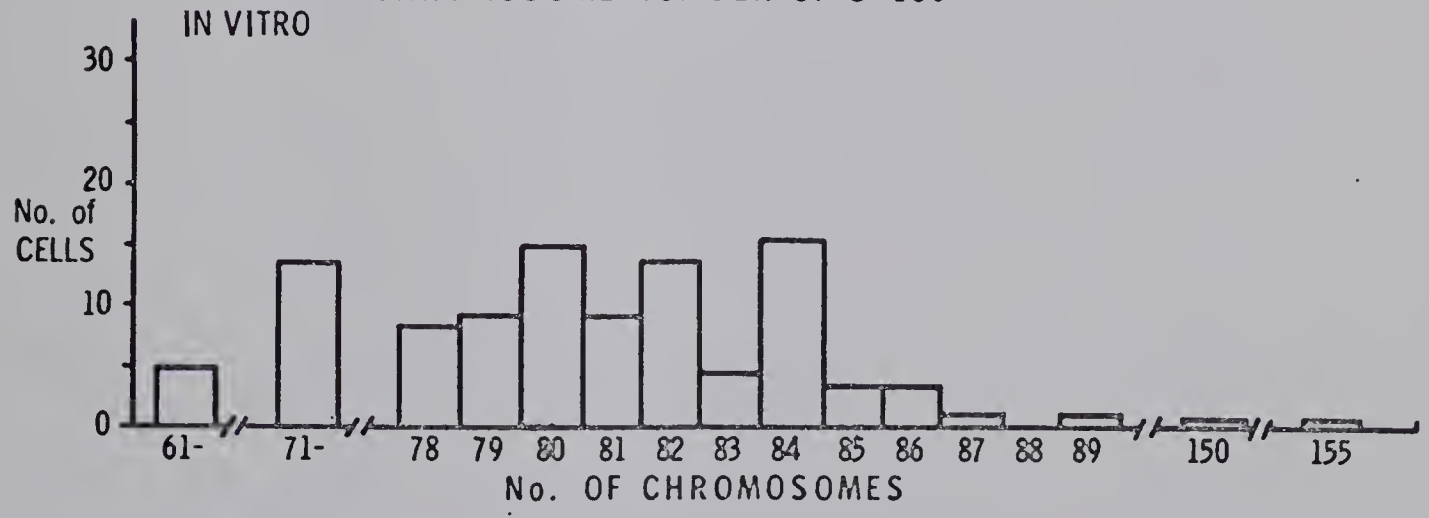
Fig. 2 Graphs illustrating chromosome number and range of S-180 (Foley) *in vitro* and *in vivo* in the C3H mouse.

CHROMOSOME NUMBER OF L-5178Y





CHROMOSOME NUMBER OF S-180



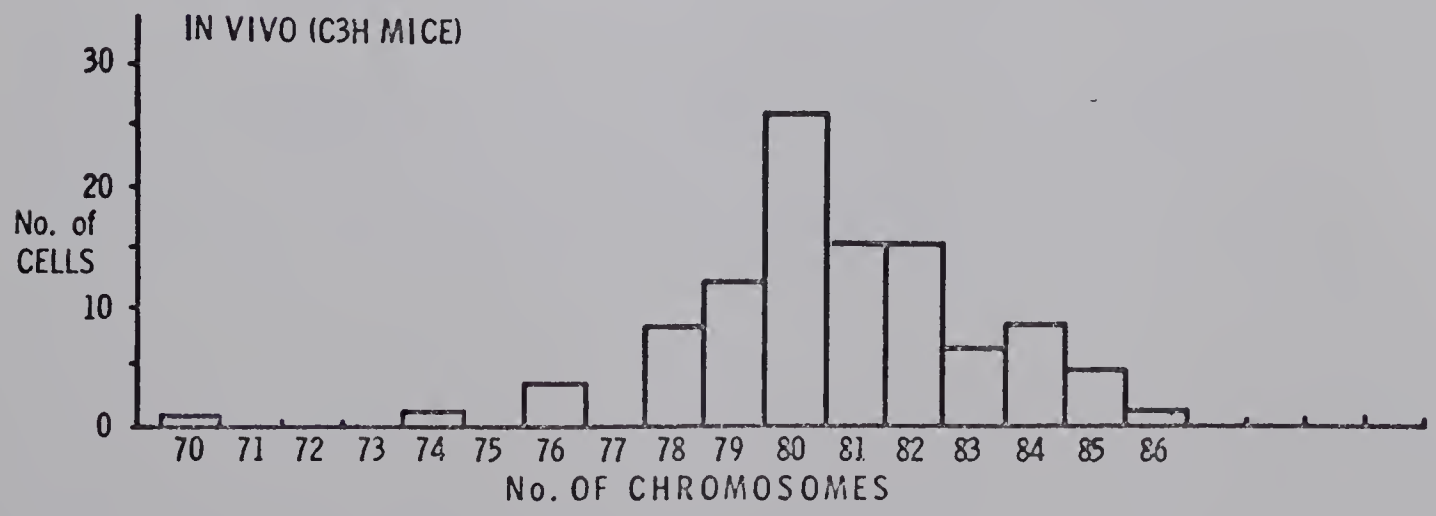
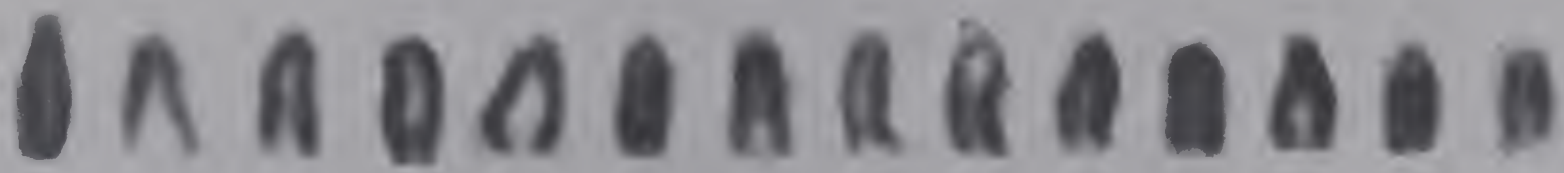
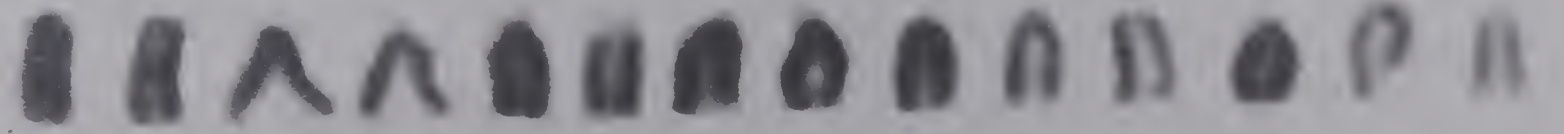


Fig. 3 Representative karyotype of the L-5178Y lymphoblast cell line. [41 acrocentric chromosomes (s)]





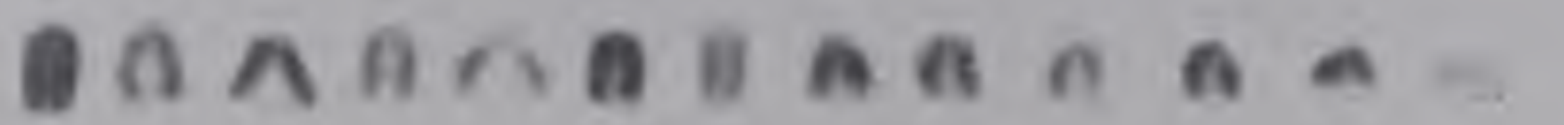
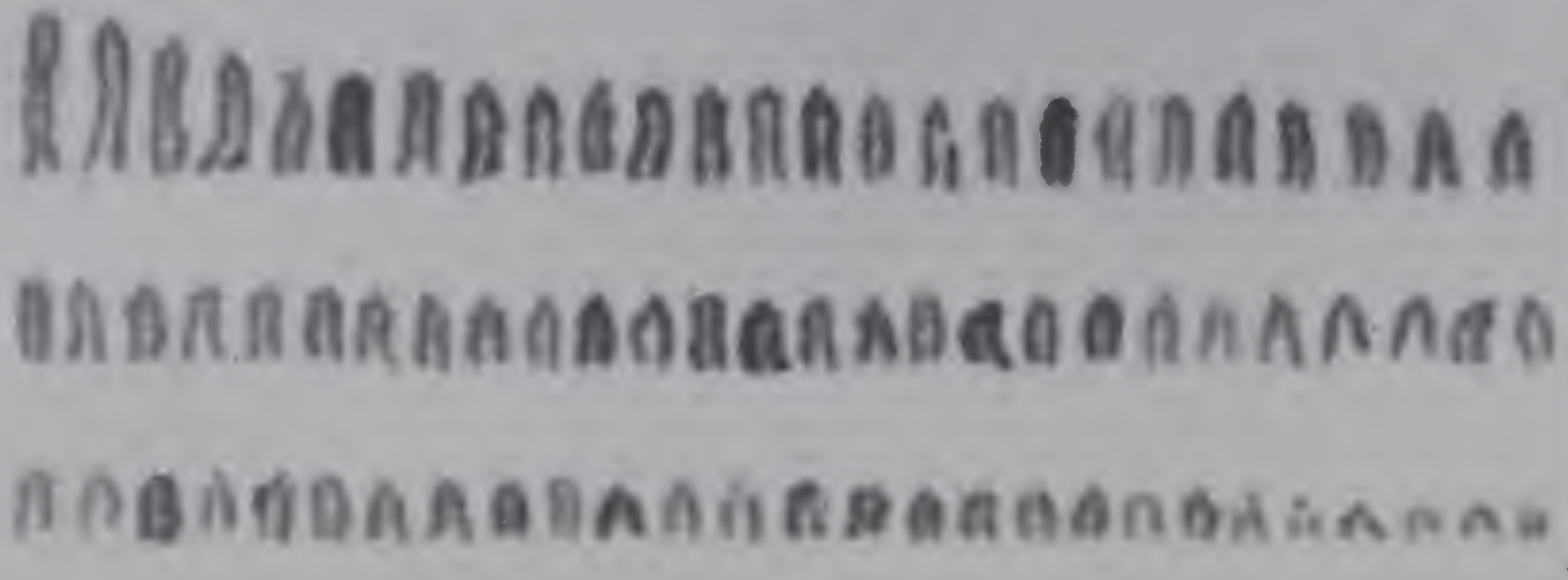


Fig. 4 Representative karyotype of the Sarcoma 180 (Foley) cell line. [84 chromosomes (s) - three metacentric, two minute]

GROUP I

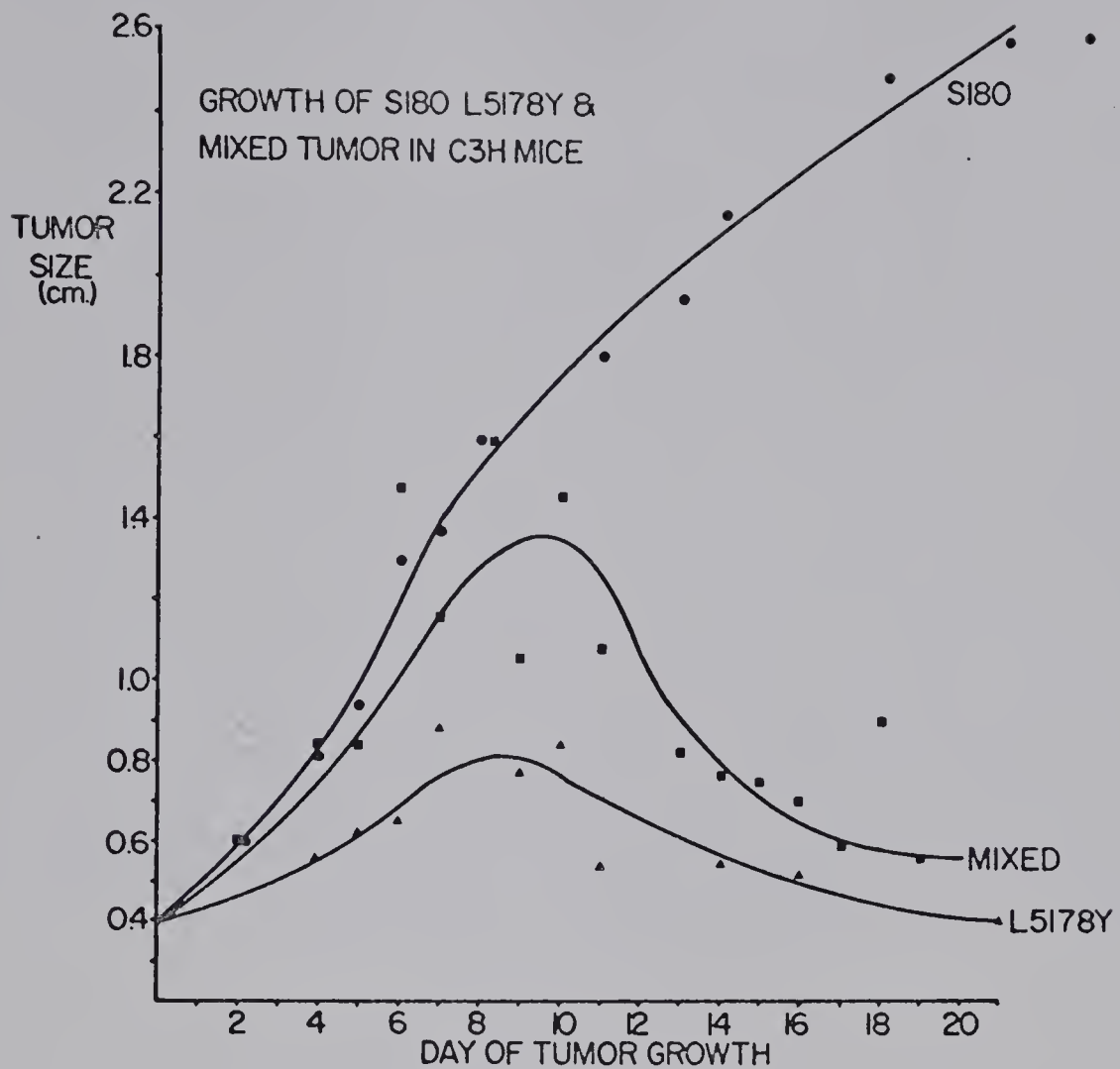
GROUP II



GROUP III

Fig. 5 Growth curves of S-180 (Foley), L-5178Y, and mixed tumors in C3H mice.

Fig. 6 Growth curves of S-180 (Foley), L-5178Y, and mixed tumors in BDF mice.


GROWTH OF S180 L5178Y &
MIXED TUMOR IN C3H MICE
S180
MIXED
L5178Y
TUMOR SIZE (cm.)
2.6
2.2
1.8
1.4
1.0
0.8
0.6
0.4
2
4
6
8
10
12
14
16
18
20
DAY OF TUMOR GROWTH

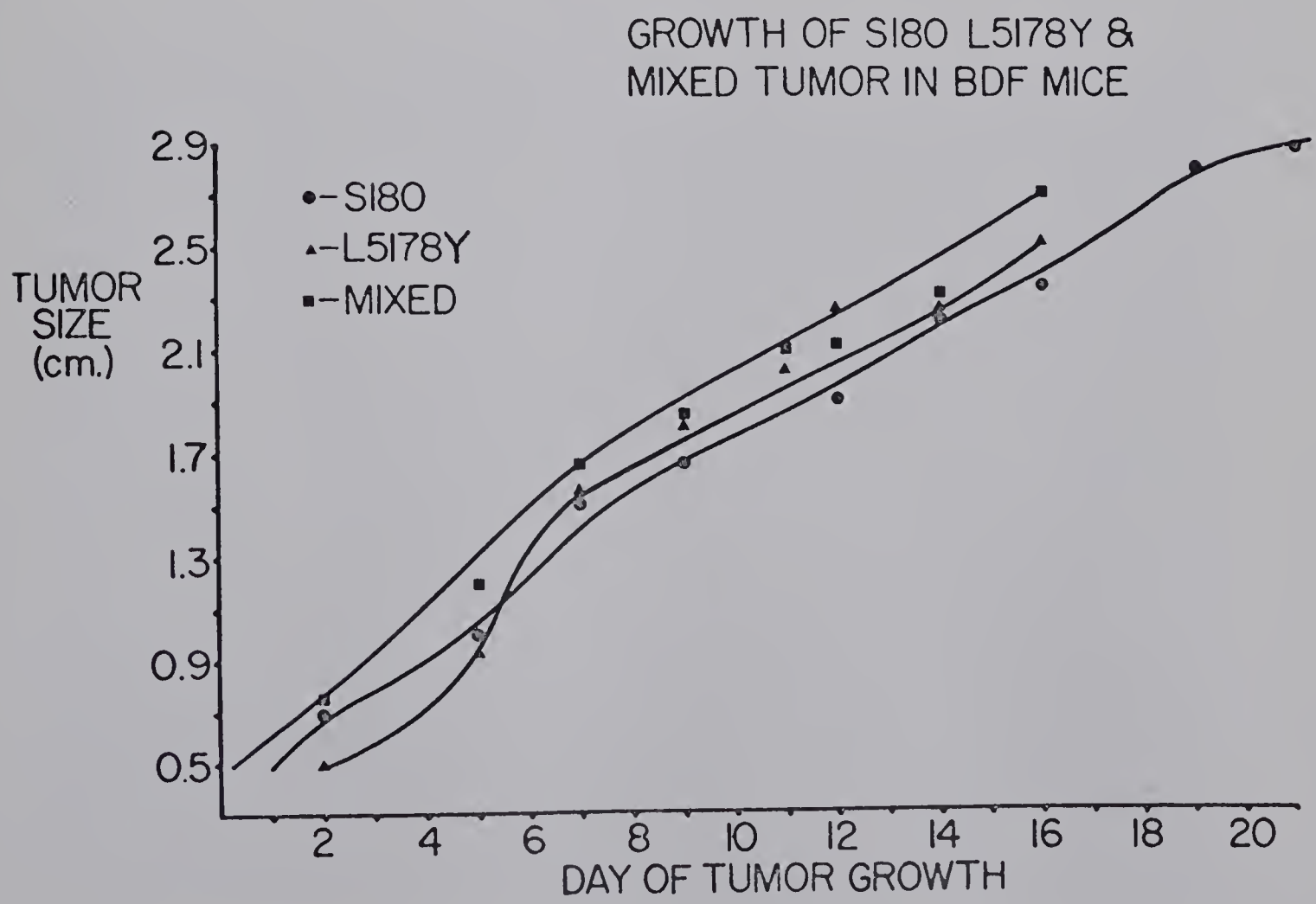
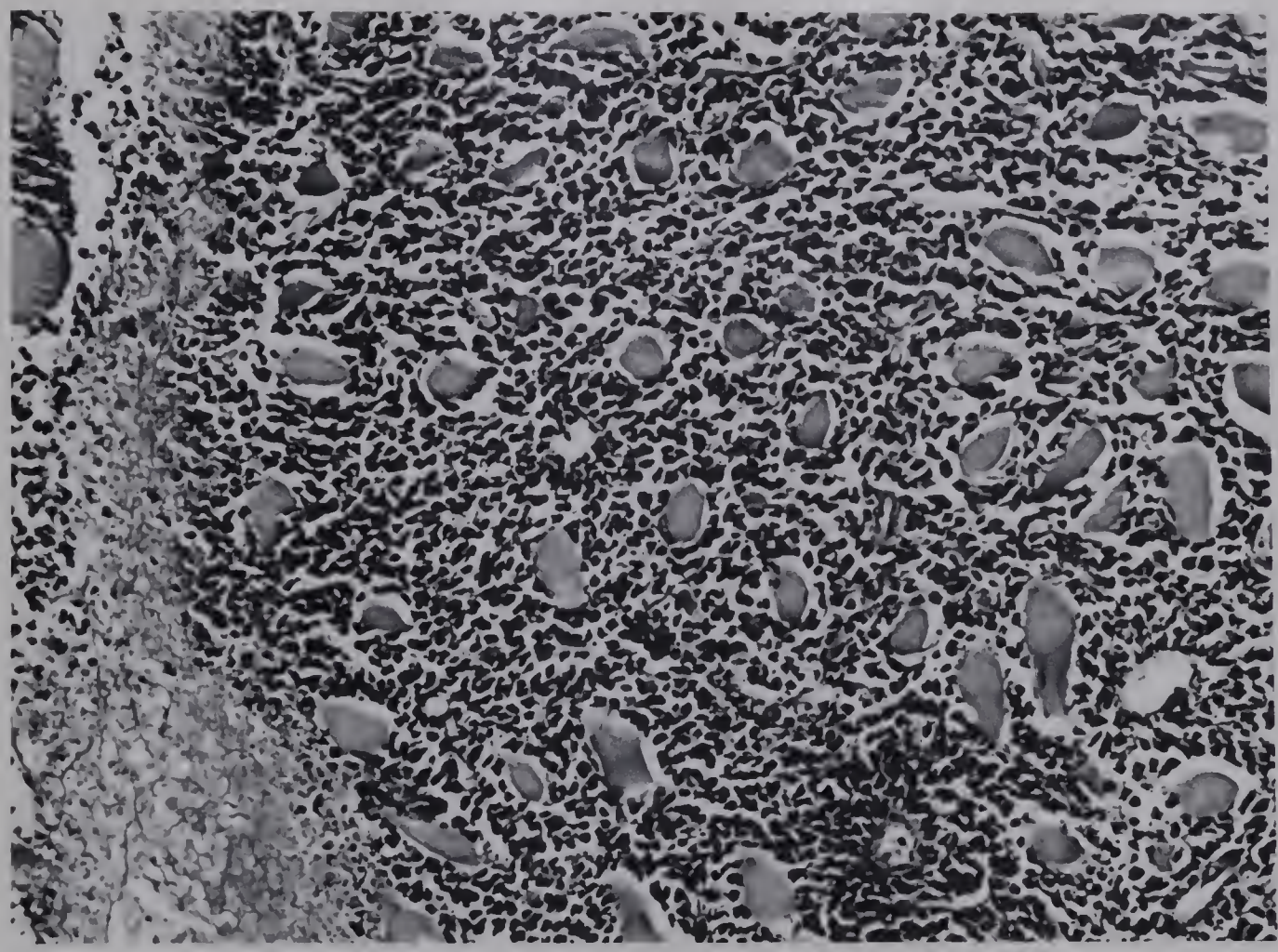

GROWTH OF S180 L5178Y &
MIXED TUMOR IN BDF MICE
●-S180
▲-L5178Y
■-MIXED
TUMOR SIZE (cm.)
2.9
2.5
2.1
1.7
1.3
0.9
0.5
2
4
6
8
10
12
14
16
18
20
DAY OF TUMOR GROWTH

Fig. 7 Histologic section of L-5178Y tumor in C3H mouse at one week. A sheet of small dark lymphoblast-like cells is invading skeletal muscle. An area of necrosis is shown on the left. (125X)

Fig. 8 Histologic section of S-180 (Foley) tumor in C3H mouse at one week. Fibroblast-like cells are shown in an unorganized pattern of whorls and strands invading skeletal muscle. (125X)



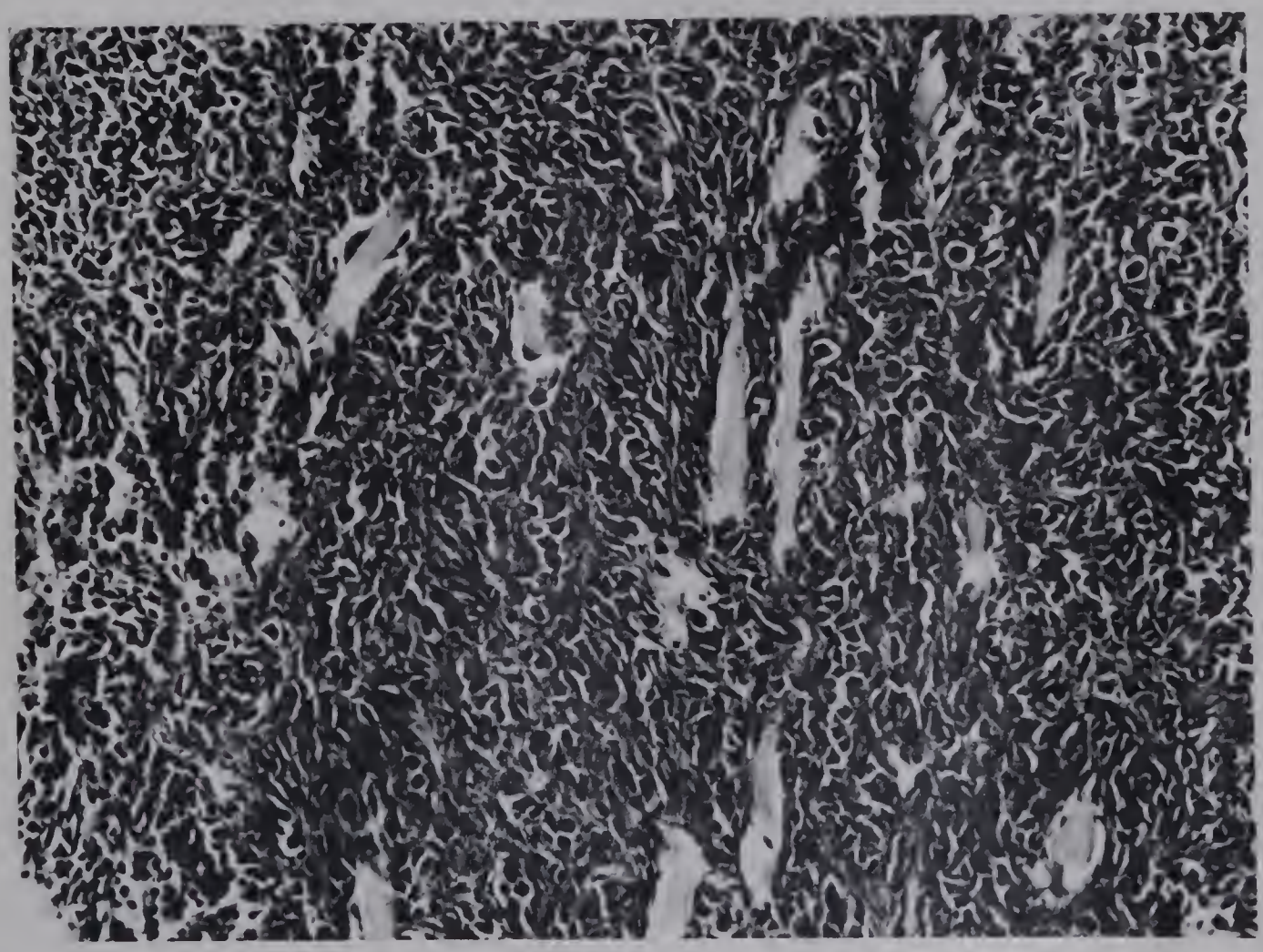
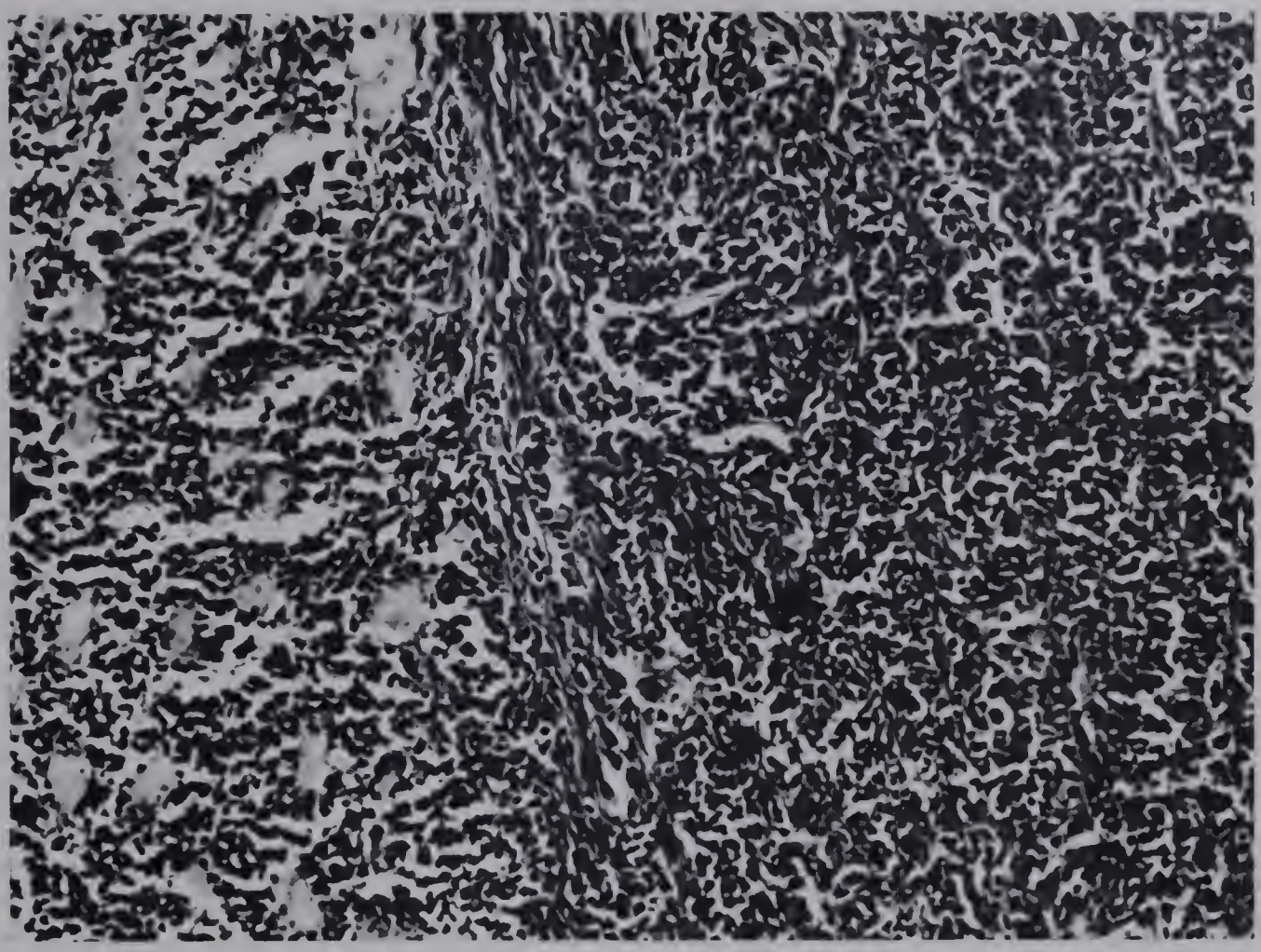


Fig. 9 Histologic section of a mixed solid tumor in the C3H mouse at one week. Fibroblast-like cells of the S-180 (Foley) are centrally located, while the L-5178Y lymphoblast-like cells are predominantly on the left, but also appear on the right. (125X)

Fig. 10 Histologic section of a mixed solid tumor in the C3H mouse at two weeks. Strands of fibroblast-like cells can be seen against a background of small round lymphoblast-like cells. Areas of necrosis are present. (125X)



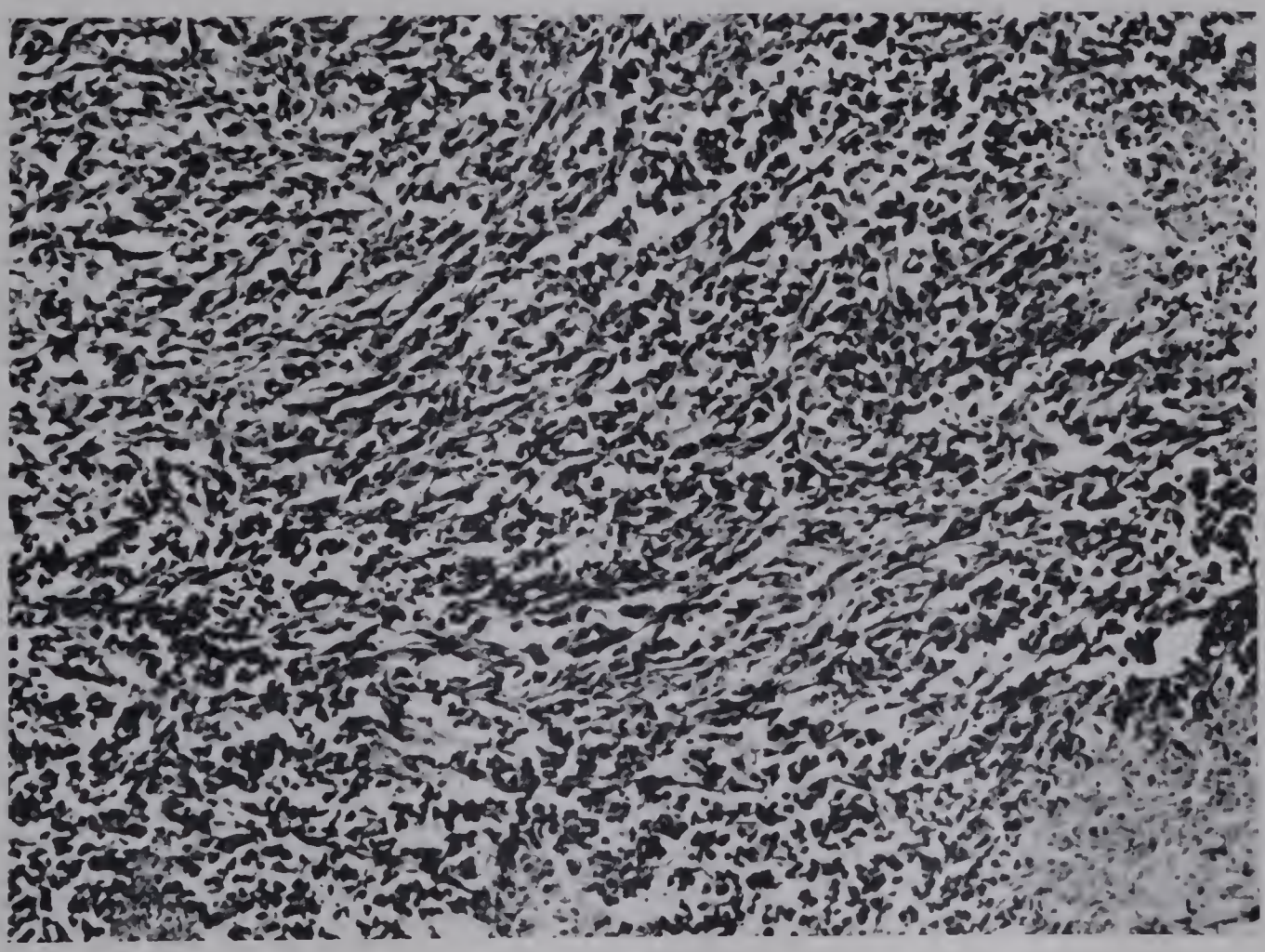
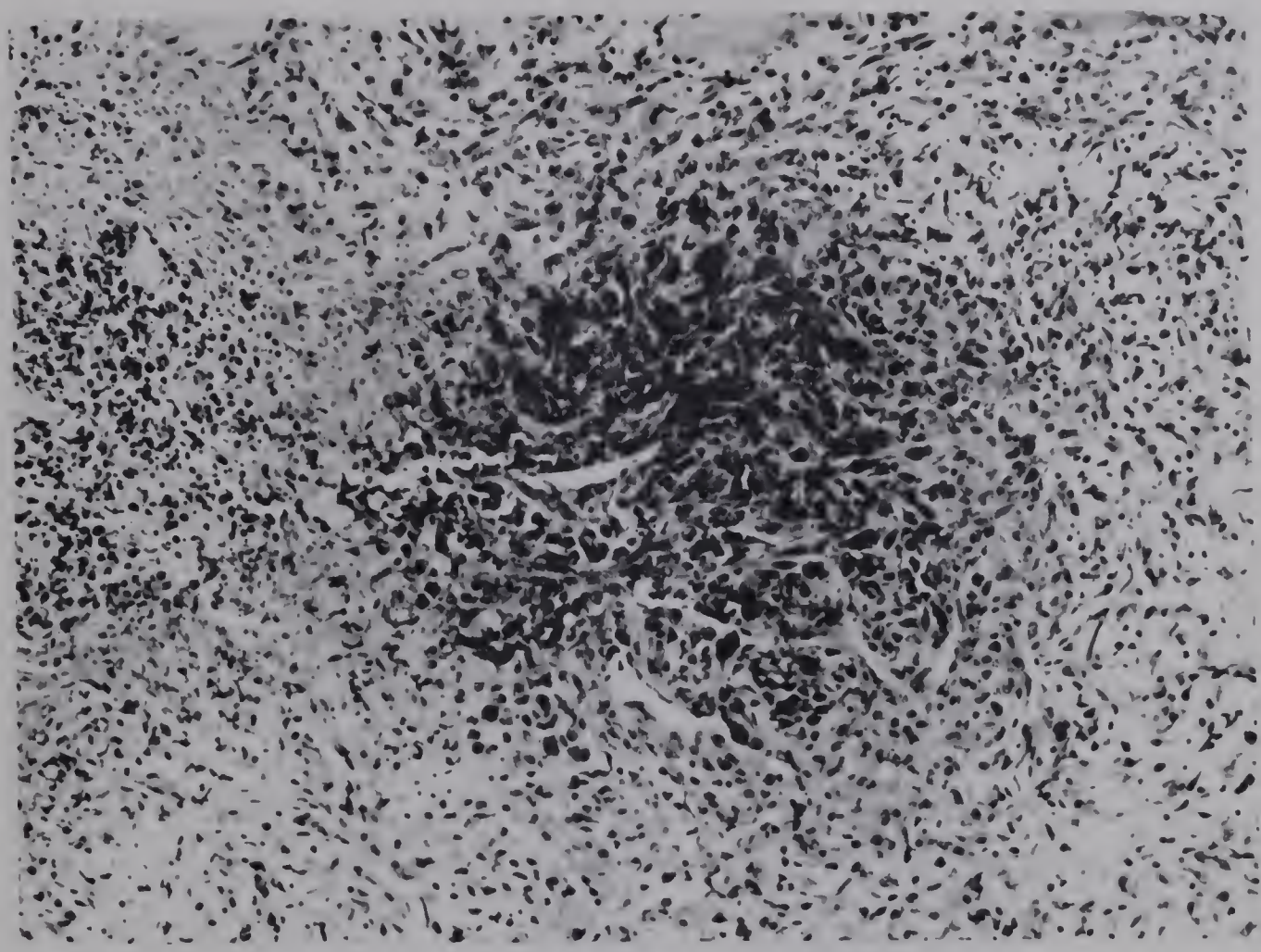


Fig. 11 Histologic section of a mixed solid tumor in the C3H mouse at three weeks. An isolated nest of large fibroblast-like cells is surrounded by a loose arrangement of lymphoblast-like cells. Areas of necrosis are developing on the left. (125X)

Fig. 12 Enlarged section of the central cellular area in Fig. 11 to show the presence of binucleate and multinucleate cells as they appear in the mixed solid tumor. The binucleate cells may represent hybrid cell precursors. (450X)



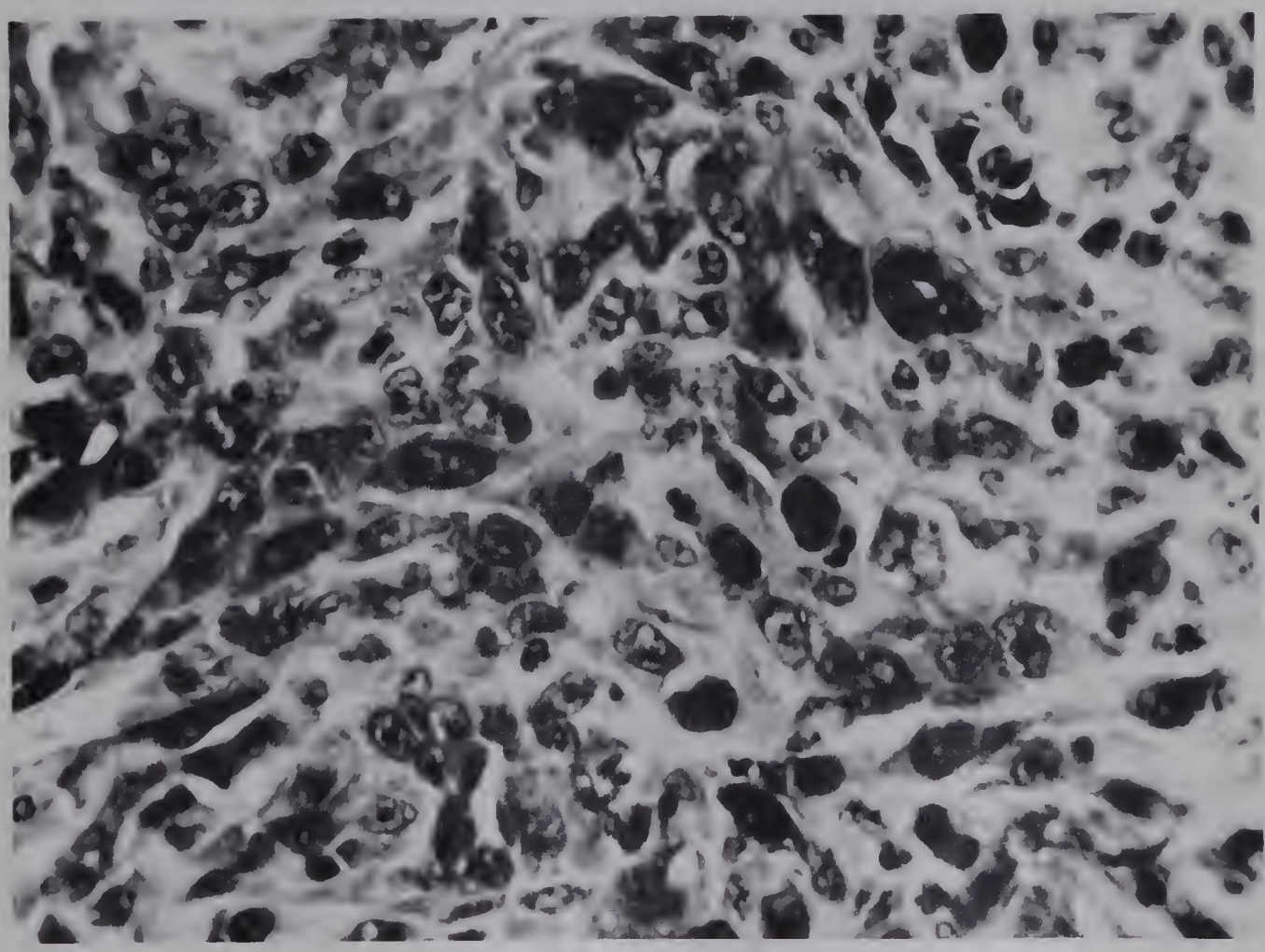


Fig. 13 Histogram illustrating the hybridization (somatic cell fusion) of Sarcoma 180 (Foley) and L-5178Y *in vivo* in the C3H mouse.

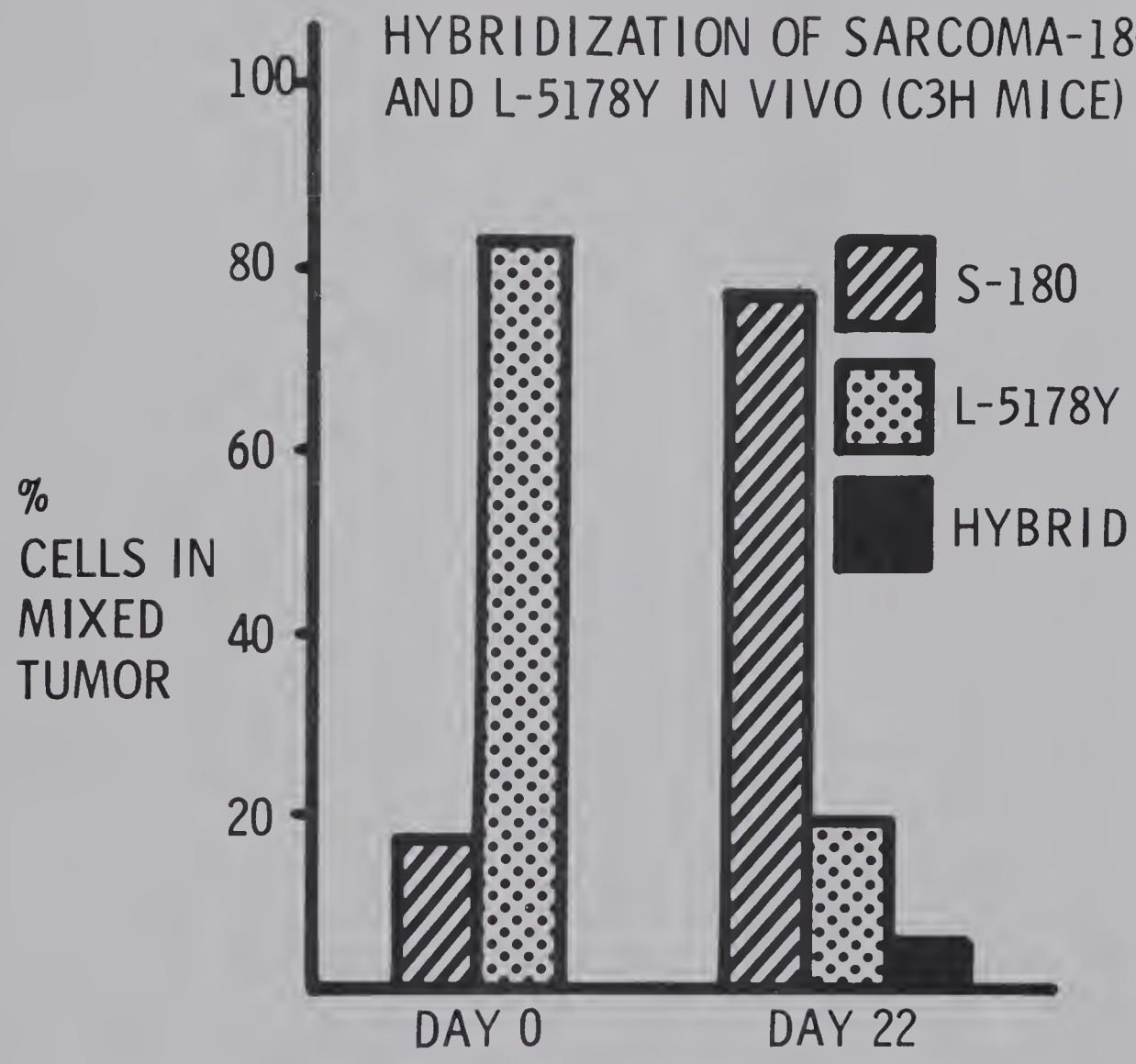

HYBRIDIZATION OF SARCOMA-180
AND L-5178Y IN VIVO (C3H MICE)
%
CELLS IN
MIXED
TUMOR
100
80
60
40
20
S-180
L-5178Y
HYBRID
DAY 0
DAY 22

Fig. 14 Karyotype of the hybrid cell shown in the insert with four metacentric marker chromosomes (large, medium, and two small) and a chromosome number of 150 (s S-180 + 2s L-5178Y).

GROUP I

GROUP II

GROUP III

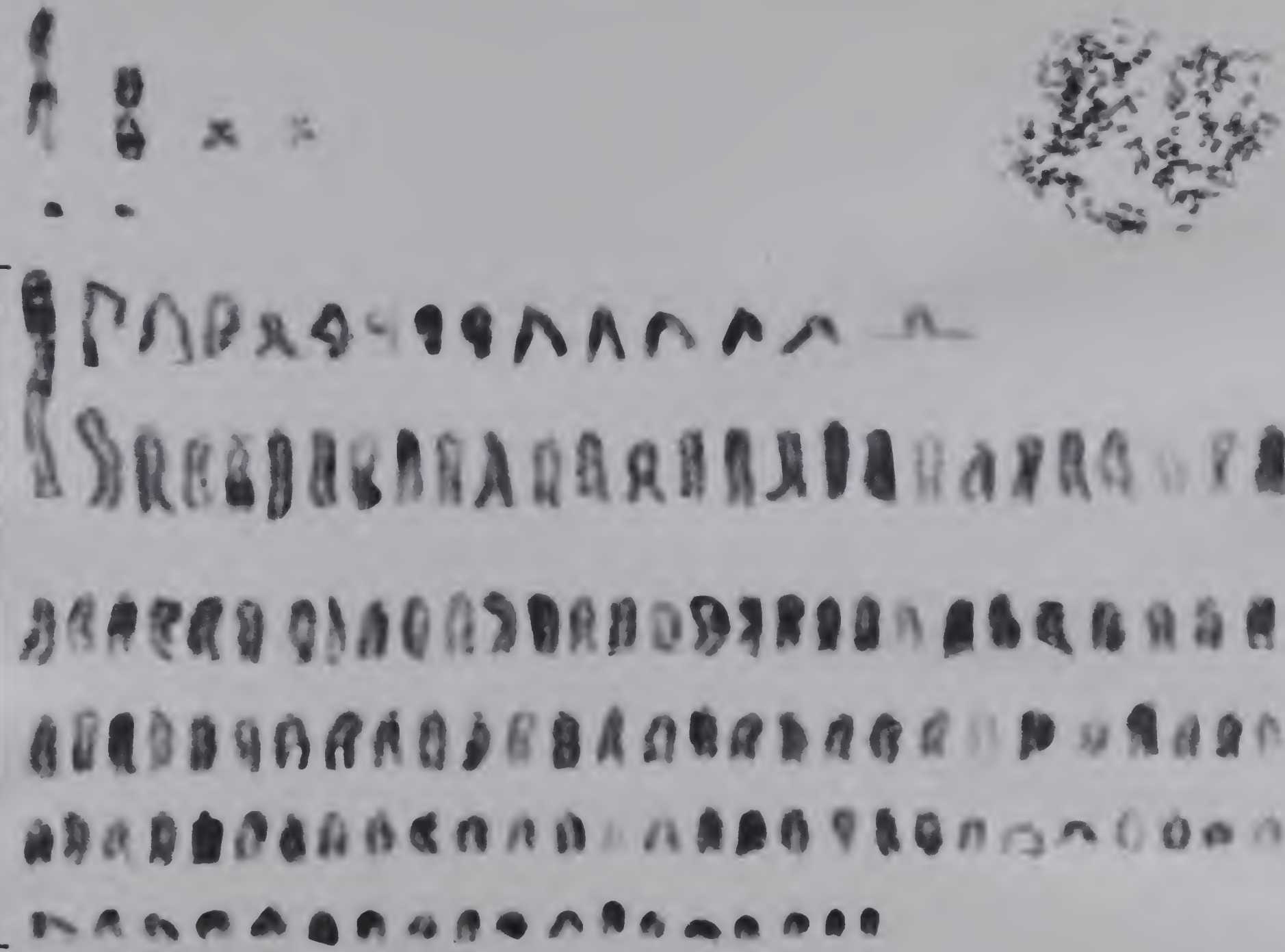


Fig. 15 Representative karyotype of a hybrid cell with three metacentric marker chromosomes (large, medium, small) and a chromosome number of 125 (s S-180 + s L-5178Y).

GROUP I

GROUP II –

GROUP III

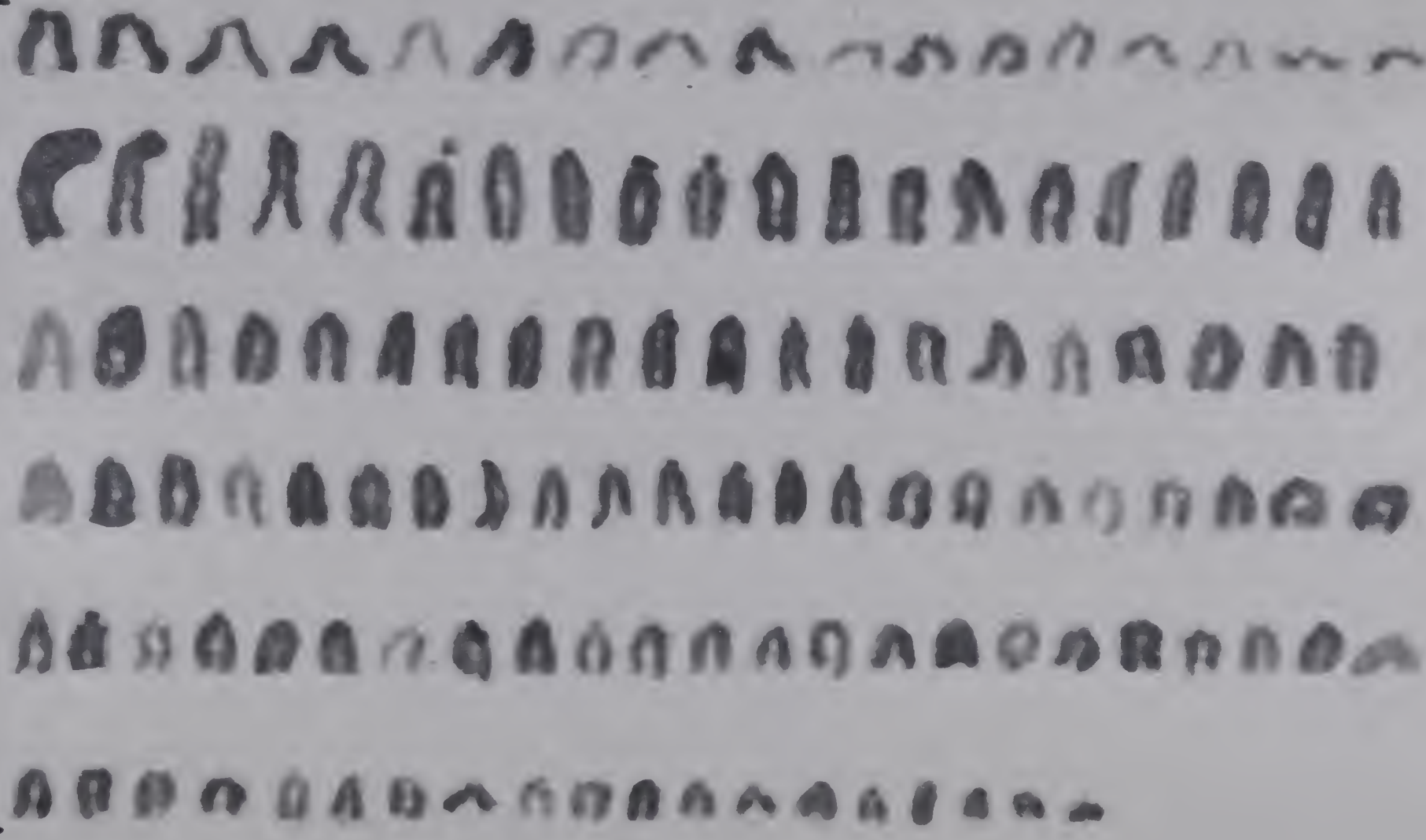


Fig. 16 Hybrid cell with three metacentric marker chromosomes (large, medium, small) and a chromosome number of 141 (s S-180 + 2s L-5178Y).

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